CHARACTERISATION OF THE T-CELL PROLIFERATION IN MALIGNANT CATARRHAL FEVER

Thesis submitted for the Degree of Doctor of Philosophy

by

Alexandra Schock

University of Edinburgh

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Declaration

I declare that all the work presented in this thesis has been composed and performed by myself. Contributions to the work of this thesis by colleagues are fully acknowledged in the text.

This work had not been, and is not currently being submitted for candidature for any other degree.

Alexandra Schock

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Abbreviations

AAS	amino acid sequence
Ag	antigen
AHV-1	*Alcelaphine herpesvirus-1*
AN	accession number for the nucleic acid database
ANOVA	analysis of variance
APC	antigen presenting cells
APS	ammonium persulfate
BHV-1	*Bovine herpesvirus-1*
bov	bovine
brIL-2	recombinant bovine IL-2
BVD	Bovine viral diarrhoea
BVDV	Bovine viral diarrhoea virus
cDNA	complementary DNA
Con A	Concanavalin A
cpm	counts per minute
Cs A	Cyclosporin A
CTLL	cytotoxic T-lymphocyte line
DEPC	di-ethyl pyrocarbonate, RNAse inhibitor
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EBV	*Epstein-Barr virus*
Fab	antigen binding portion of a antibody
FACS	fluorescent activated cell sorter
FBS	foetal bovine serum
GALT	gut associated lymphoid tissue
HipHV-1	*Hippotragine herpesvirus-1*
hPMN	heterophilic polymorphonucleated granulocytes
hrIL-2	recombinant human IL-2
HVS	*Herpesvirus saimiri*
IEL	intra epithelial leukocytes

IF	immunofluorescence
IFN	interferon
IIF	indirect immunofluorescence
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
LA	lymphoid cell accumulation
LAK	lymphokine activated killer
LC	lymphocytes
LGL	large granular lymphocytes
mAb	monoclonal antibody
MALT	mucosal associated lymphoid tissue
MCF	malignant catarrhal fever
MHC	major histocompatibility complex
MOPS	3-N-morpholino-propano-sulfonic acid
NAS	nucleic acid sequence
OHV-2	*Ovine herpesvirus-2*
PALS	periarteriolar lymphoid sheath
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PHA	phytohaemagglutinine
PLNC	cells derived from popliteal lymph node
PM	post mortem
PMN	polymorphonucleated granulocytes
PPD	bovine tuberculin purified protein derivative
RNA	ribonucleic acid
RT	reverse transcription
SA-MCF	sheep-associated MCF
SDS	sodium dodecyl sulphate
sem	standard error of the mean

SHV-1	*Suid herpesvirus-1*
TCGF	T-cell growth factor (old name for IL-2)
TEMED	N,N,N',N',-tertramethylenediamine
Th	T- helper cell
Th_1	T- helper cell type 1
Th_2	T- helper cell type 2
T_m	melting temperature
TNF	tumour necrosis factor
Tris	tris(hydroxymethylene)methylamine
UV	ultraviolet
WA-MCF	wildebeest-associated MCF

Abstract

Malignant catarrhal fever (MCF) is a lethal lymphoproliferative disorder of cattle and deer caused by infection with either Alcelaphine herpesvirus - 1 (AHV-1) or Ovine herpesvirus - 2 (OHV-2). The disease can be transmitted experimentally to rabbits. Despite intensive studies, only very little viral expression can be detected in lesions. It was therefore proposed that the viruses induce an interleukin-2 (IL-2) hyperproduction which is responsible for the lymphoid cell hyperplasia observed.

In an initial comparative study, it was shown that all three viruses (AHV-1, OHV-2 and another bovid γ-herpesvirus: Hippotragine herpesvirus- 1 (HipHV-1) induced similar hyperplasia of lymphoid organs and accumulations of mononuclear cells in non-lymphoid organs in rabbits. However, the different viruses affected preferentially certain lymphoid organs. It was observed that the lymphoid cells in non lymphoid tissues were $CD43^+$ T-cells which showed evidence of *in situ* multiplication. A more detailed phenotypic analysis of splenocytes and lymph node cells in AHV-1 infected rabbits showed that the overall proportions of CD43, CD5, CD4, CD8 and B-cells were not altered, but that $CD5^+$ and $CD8^+$ cells were significantly enlarged.

The hyperplasia of lymph node cells was also investigated by examining the growth characteristics of freshly explanted lymph node cells from AHV-1 infected rabbits. The lymph node cells had a higher thymidine uptake respective to control cultures in the first 24 hours. IL-2 increased viability and thymidine uptake and Con A did not stimulate these cells as expected. Very little IL-2 activity could be detected in supernatants from short term cultures using the CTLL-based bioassay. To establish a RT-PCR/immunoblot for the detection of rabbit IL-2 mRNA, IL-2 cDNA from Con A stimulated rabbit lymphocytes was cloned and partially sequenced. IL-2 transcripts could be detected in lymphoid cells from pyrexic rabbits infected with AHV-1 and in cells from control rabbits. Furthermore, it was shown that lymphoblastoid cell lines (LCL) derived from MCF-affected cattle did not transcribe IL-2. These data lead to the conclusion that IL-2 is involved in the acute state of MCF, but does not have a central role in the pathogenesis.

Further characterisation of IL-2 dependent LCL showed that they responded weakly to Con A, were inhibited by Cyclosporin A and transcribed constitutively IL-4, IL-10, INFγ and TNFα, whereas no IL-1β mRNA could be detected. These data together with the results derived from the short term cultures of lymph node cells from AHV-1 infected rabbits clearly show that MCF inducing viruses alter the behaviour of lymphoid cells. The possible interference of OHV-2 and AHV-1 with transductional pathways, the expression of IL-2R and the activation of self-reacting lymphocytes is discussed.

Chapter 1

General Introduction

1.1. Introduction

The main purpose of this work was to investigate the immunopathology of malignant catarrhal fever (MCF), a pathological syndrome of cattle, deer and other large ruminants induced by a group of bovine γ-Herpesvirinae. The working hypothesis was based on the assumption that the pathogenesis of MCF is due to a polyclonal, non specific, benign T-cell proliferation induced by excessive interleukin-2 (IL-2) production. The literature review will therefore focus on the disease itself, on the biological roles of IL-2 and the interaction of other Herpesviruses with the immune system.

Figure 1. 1 Typical appearance of a bull (BJ 1004) affected with the head and eye form of MCF. Note the ocular, nasal and oral discharge and the closed eyes.

1.2. Malignant Catarrhal Fever: an extraordinary disease

Synonyms: Malignant Head Catarrh, Snotsiekte, Bösartiges Katarrhalfieber (BKF), Coryza gangrenosa

Malignant catarrhal fever (MCF) is a pathological syndrome of Bovidae and Cervidae characterised by high fever and generalised lymphoadenopathy, defined by the characteristic histopathological lesions. The disease is not transmitted naturally between MCF-affected animals, but from other ruminant species, (wildebeest, sheep and probably others) which, following an acute asymptomatic infection, are latently infected for life and do not develop disease. This epidemiological feature led to the identification of the aetiological agents: *Alcelaphine herpesvirus-1* (AHV-1) and *Ovine herpesvirus-2* (OHV-2), two bovid γ-Herpesvirinae. Because of its unusual epidemiology, interesting aetiology, and fascinating pathogenesis, the study of MCF is of value for the understanding of more general principles in zoology, virology and immunology.

1.2.1 Epidemiology

MCF is a disease recognised world-wide which is limited only by the presence of the **disease-susceptible species** (species which can be affected by MCF, but are unable to transmit the disease) and the **natural hosts** (latently infected species which do not develop MCF) in the same environment. An equivalent epidemiology has been described for the closely related *Herpesvirus saimiri* (HVS) and *H. atalese* in which different species of monkeys are the natural and disease-susceptible hosts (reviewed by Fleckenstein and Desrossiers, 1982). Animals probably contract the disease following environmental contamination by the natural host. In Africa, wildebeest are the natural host of AHV-1, whereas world-wide the other source of infection is presumed to be the sheep. This peculiar epidemiological pattern and the poorly

defined aetiology led to the definition of two generally recognised forms based on the involvement of the natural hosts:

I. African or Wildebeest-associated Malignant Catarrhal Fever (WA-MCF)
II. European or Sheep-associated Malignant Catarrhal Fever (SA-MCF)

In cattle, the disease is generally of high mortality and low morbidity (Plowright, 1968). However, epidemics have been described (Piercy, 1954b; Pierson *et al.,* 1973; James *et al.,* 1975; Orsborn *et al.,* 1977; Harris *et al.,* 1978; Mare, 1977; Kalunda *et al.,* 1982; Foster, 1983; Snowdon, 1985; Saroja *et al.,* 1987). This concept of high mortality may have to be reviewed in the light of the new diagnostic methods available, in that until now histopathology has provided the only definitive method of diagnosis. Animals which survived infection could not be identified (Chapter 1.2.4).

1.2.1.1 Wildebeest-associated Malignant Catarrhal Fever (WA-MCF)

Since the early days of European settlements in Africa, the correlation between calving season of wildebeest and the incidence of a "snot sickness" or "snotsiekte" in cattle was noticed (Cumming, 1850; Mettam, 1923). The incidence of MCF in areas where many wildebeest and cattle graze together can be as high as 12% (Kalunda *et al.,* 1982). The aetiological agent was identified as a virus (Plowright *et al.,* 1960), subsequently designated AHV-1 (Reid *et al.,* 1975), which is present in the free living population of wildebeest (*Connochaetes taurinus and C. gnu*) and induces the disease in cattle (Chapter 1.2.2.1). In zoological collections, WA-MCF has also been observed outside the African continent (Castro *et al.,* 1984, Meteyer *et al.,* 1989).

1.2.1.2 Sheep-associated Malignant Catarrhal Fever (SA-MCF)

The role of domestic sheep (*Ovis aries*) as the source of SA-MCF was based on epidemiological observation (Goetze and Lies, 1930; Magnusson, 1940; Piercy, 1954a; Orsborn *et al.,* 1977; Reid and Buxton, 1984). However, while experimental transmission from sheep to cattle failed on several occasions (Mettam, 1923; Blood

et al., 1961), some flocks of sheep are particularly "pathogenic" for cattle (Goetze and Liess, 1930, De Kock and Neitz, 1950, Piercy, 1954a). Recently, the identification of OHV-2 (Chapter 1.2.2.2) confirmed the connection between the inapparently infected sheep and disease in cattle and deer (Baxter *et al.,* 1993).

1.2.1.3 Natural Transmission of the Infection

Although most transmission studies have been done using WA-MCF, since it is possible to isolate the virus directly, serological and molecular studies indicate a similar epidemiology for SA-MCF. The experimental transmission of both forms of MCF is discussed in chapters 3 and 8.

The **natural hosts**, wildebeest and sheep, become latently infected with the MCF-inducing virus and the only sign of infection is seroconversion. On the basis of serological evidence, nearly every adult wildebeest and sheep are infected with AHV-1 (Plowright, 1967) or OHV-2 (Rossiter, 1981) respectively. It would appear that virus is acquired at an early age and all individuals seroconvert within the first 6 months of life (Mushi *et al.,* 1981a; Barnard, 1990, Li *et al.,* 1994a, 1995a). The AHV-1 can be transmitted by the intrauterine route (Plowright, 1965 a, b, Mushi *et al.,* 1980a, b) and productive infection of neonates occurs as maternal antibodies decline (Mushi *et al.,* 1981c; Li *et al.,* 1995b).

The viruses are normally cell-associated in the natural host and in MCF-affected animals (Plowright, 1965a). However, virus spreads rapidly and highly efficiently in populations of the natural host which implies abundance excretion of contagious presumably cell-free virus. It was hypothesised that the cell-free form of the virus is shed into the environment during the acute productive phase of infection and in situations of immunosuppression (Plowright, 1965b). Cell-free AHV-1 has been detected in nasal and ocular secretions of young and immunosuppressed wildebeest (Rweyemamu *et al.,* 1974; Mushi *et al.,* 1980 a, b, 1981c), whereas it could not be isolated from the placenta (Rossiter *et al.,* 1983a).

The **disease-susceptible species,** or dead end hosts, which develop MCF, usually do not transmit the disease to other individuals (Plowright, 1968). It is thought that horizontal transmission is impossible, because of the lack of cells in the cattle and

deer, which are competent to produce cell-free virus and, therefore, infective virus (Mushi *et al.*, 1981c). The disease-susceptible animals are infected in the field during the peri-parturient period of the natural host. The virus is released into the environment either by the immunosuppressed mothers or by their acutely infected offspring (Daubney and Hudson, 1936; Plowright, 1965b; Harris *et al.*, 1978; Li *et al.*, 1995b). The only evidence for transmission of MCF from one susceptible animal to another is the observation that foetuses *can* become infected *in uterus* from their mothers which survived an episode of MCF (Plowright *et al.*, 1972; Barnard, 1990). Since the source of the disease can not always be easily identified, it has been proposed that a vector could be involved in the transmission of the disease. However, no vector, such as ticks, lice and African face fly, could be identified and the levels of viraemia are too low to permit effective transmission by such a mechanism (Mettam, 1923; Barnard, 1989, 1990). A possible explanation could be the presence of other, as yet unidentified, natural hosts (Chapter 1.2.2.3).

1.2.1.4 The disease: which species can get it ?

The disease has been described affecting a variety of species of the Family **Bovidae** (Subfamilies: Bovinae, Caprinae, Tragelaphinae, Antilopinae and Hippotraginae) and **Cervidae** (Subfamilies: Cervinae, Alcinae, Rangiferinae and Odocoilinae) (Table 1.1).

The disease has mostly been recognised, for obvious reasons, in economically important species, such as domestic cattle, Bali cattle and domestic buffalo. For the same reason the disease was detected in the Axis deer and red deer, when these species were managed intensively. Other species, in which the disease has been recognised, have mostly been housed in zoos and, therefore, closely supervised and exposed to relatively intimate contact with the natural hosts which might not be part of their natural habitat. It is also noteworthy that farmed red deer are very susceptible to SA-MCF, whereas wild deer do not appear to become infected despite apparently sharing the same habitat with sheep throughout large tracts of land in Scotland.

The epidemiological data do not indicate a clear phylogenetic pattern between the natural hosts and the species which can be affected by MCF (Table 1.1). It was in fact shown that the most susceptible animals are Pere David's deer, Bali cattle and red deer, whereas zebu and European cattle seem to be relatively resistant to the disease (Daniels *et al.,* 1988; Young *et al.,* 1988; Reid and Buxton, 1989). Since MCF occurs when species which do not normally share the same habitat are brought into contact, it could be concluded that animals which naturally co-habit with sheep and wildebeest have developed natural resistance. One species of deer which appears to be naturally resistant to SA-MCF is the fallow deer (*Dama dama*), which, together with domestic sheep, had its origin in the Mediterranean basin (Reid, personal communication).

It has been speculated that sheep, being identified as the natural host of OHV-2, may be affected by clinical disease. Naturally occurring disease has not been reported, but pathologically similar diseases of unknown aetiology have been described under various circumstances (Kalunda *et al.,* 1980; Schmitz and Grumbein, 1981, Buxton *et al.*, 1985; Rae, 1995). Plowright (1981) tried to infect sheep with AHV-1, but failed to recover virus, whereas Piercy (1954a) and Huck *et al.,* (1961) unsuccessfully attempted to infect sheep from diseased cattle.

SA-MCF and WA-MCF can be experimentally transmitted to **rabbits** (Daubney and Hudson, 1936; Buxton and Reid, 1984; Reid *et al.,* 1986; Buxton *et al.,* 1984) and hamsters (Buxton *et al.,* 1984; Jacoby *et al.,* 1988 a, b), whereas only WA-MCF is transmissible to rats (Jacoby *et al.,* 1988a, b) and Guinea pigs (Jacoby *et al.,* 1988a, b).

Table 1. 1 Species of Bovidae in which MCF has been described

Subfamily[1]	Species	Common Name	WA[2]	SA[3]	References
Bovinae	*Bos taurus*	European Cattle	+	+	Goetze, 1930; Mettam, 1923;
	Bos indicus	Zebu Cattle	+	+	Plowright, 1981;
	Bos juvanicus	Bali Cattle	+	+	Young *et al.,* 1988;
	Bos gaurus	Gaur	+		Castro *et al.*, 1982; Hatkin, 1980
	Bubalus bubalis	Domestic Buffalo		+	Vanselow, 1980; Hoffmann *et al.,* 1984;
	Bison bison	American Bison		+	Pierson *et al.,* 1974; Ruth *et al.,* 1977;
	Bison bonasus	European Bison	+	? [5]	Balsai, 1973; Heuschele *et al.*, 1984;, Straver and van Bekkum, 1979,
	Bosephalus tragocamelus	Nilgai	+		Roken and Borklund, 1974; Heuschele *et al.*, 1984;
Caprinae	*Ovis aries*	Domestic Sheep	+	+	[4]Kalunda *et al.,* 1980; Schmitz and Grumbein, 1981; [4]Buxton *et al.*, 1984; Rae, 1994;
	Capra aegagrus	Domestic Goat		?	Altmann, 1973; Heuschele *et al.*, 1984;

continued →

Table 1.1 continued

Subfamily[1]	Species	Common Name	WA[2]	SA[3]	References
Tragelaphinae	*Tragephalus strepsiceros*	Greater Kudu	+		Castro *et al.*, 1982; Boever and Kurka, 1974;
	Tragephalus spekei	Sitatunga	+		Roken and Bjorklund, 1974;
	Tragephalus scriptus	Bushbuck	+		Heuschele *et al.*, 1984
	Taurotragus eurycerus	Bongo	+	+	Heuschele *et al.*, 1984
	Taurotragus oryx	Eland	+		[4]Plowright, 1981;
Antilopinae	*Antilope cervicapra*	Blackbuck	+		Heuschele *et al.*, 1984
	Gazella leptoceros	Slender-horned gazelle	+		Heuschele *et al.*, 1984
Hippotraginae	*Oryx leucoryx*	Arabian Oryx	+		Heuschele *et al.*, 1984
	Hippotragus equinus	Roan Antilope	?	?	Gulland *et al.*, 1989

[1] classification by Miyamoto *et al.*, 1993; [2] WA-MCF; [3] SA-MCF; [4] experimentally induced; [5] in zoological collection and, therefore, no clear epidemiology

Table 1. 2 Species of Cervidae in which MCF has been described

Subfamily[1]	Species	Common Name	WA[2]	SA[3]	References
Cervinae	*Cervus elaphus*	Red Deer	?	+	Oliver *et al.*, 1983, 1984, 1988; Reid *et al.*, 1979, 1986;
	Cervus nippon	Sika Deer	+	?	Wilson *et al.*, 1983; Sanford, 1977; Heuschele *et al.*, 1984;
	Cervus timorensis	Rusa Deer		+	Denholm and Westbury, 1982
	Cervus duvauceli	Barabsingha	+		Heuschele, 1984
	Axis axis	Axis Deer	+	+	Lüpke, 1906; Castro *et al.*, 1982;
	Elapharus davidianus	Pere David's Deer		+	Huck *et al.*, 1962; Tong *et al.*, 1961; Reid *et al*,1987;
Alcinae	*Alces alces*	Elk		+	Anderson, 1953; Williams *et al.*, 1984;
Rangiferina	*Rangifer tarandus*	Reindeer		?	Altmann, 1973;
Odocoilinae	*Odocoileus hemionus*	Mule Deer		+	Pierson *et al.*, 1974;
	Odocoileus virginianus	White Tailed Deer	?	+	Clark *et al.*, 1972; Castro and Daley, 1982; Whitenack *et al.*, 1981; Wobeser *et al.*, 1973; Shulaw and Ogelsbee, 1989;
	Capreolus capreolus	Roe Deer		+	Hanichen and Ernst, 1969; Reid *et al.*, 1986;

[1] classification by Miyamoto *et al.*, 1993; [2] WA-MCF; [3] SA-MCF; [4] experimentally induced; [5] in zoological collection and, therefore, no clear epidemiology

1.2.2 MCF: one syndrome and many aetiological agents

From the early days, it was understood on the basis of cattle to cattle transmission studies mainly with whole blood, that MCF is induced by a strongly cell-associated transmissible agent (Mettam, 1923; Daubney and Hudson, 1936). The definition of MCF is based on a pathological syndrome, but from epidemiological studies, it became apparent that the disease occurred under two circumstances: following contact with wildebeest, referred to as WA-MCF, and with sheep, referred to as SA-MCF (Chapter 1.2.1). Subsequent studies identified *Alcelaphine herpesvirus-1* (AHV-1) and *Ovine herpesvirus-2* (OHV-2) respectively as the aetiological agents. Other related γ herpesviruses have been identified in ruminants, but their pathogenic potential for cattle and deer is not known (Chapter 1.2.2.3).

1.2.2.1 *Alcelaphine Herpesvirus -1* and *-2* (AHV-1 and 2)

WA-MCF in cattle in Africa is caused by a γ-herpesvirus which has previously been called *Bovine herpesvirus-3* (BHV-3) (WHO/FAO, 1976) and later reclassified (Reid *et al.,* 1974, 1975, Roizman *et al.,* 1982) as *Alcelaphine herpesvirus-1* (AHV-1). The typical ether and chloroform sensitive herpesvirus which causes syncythia and intranuclear inclusion bodies in cell culture was isolated for the first time from a blue wildebeest (*Connochaetes taurinus*) by Plowright *et al.,* (1960). The virus is strongly cell-associated in its natural host and in MCF-affected animals. Only after a number of passages in tissue culture, can cell-free virus be detected at appreciable titre (Plowright 1965a; Plowright *et al.,* 1965; Reid and Rowe, 1973). The adaptation of the virus to cell culture leads to progressive loss of pathogenicity in rabbits (Handley, 1993; Handley *et al.,* 1995). The viral genome has been mapped with restriction enzymes and partially sequenced (Shih *et al.,* 1988, 1989; Bridgen *et al.,* 1989, 1990; Hsu *et al.,* 1990a; Handley *et al.,* 1995). Various viral proteins have been identified by immunoprecipitation (Adams and Hutt-Fletcher, 1990; Li *et al.,* 1995a) and open reading frames identified by sequence analysis (Ensser and Fleckenstein, 1995).

AHV-1 is antigenically distinct from BHV-1 and BHV-2, but is related to BHV-4 (Osorio *et al.,* 1985; Seal, 1989; Thiry, 1990). Restriction enzyme analysis and cross-hybridisations confirmed these findings (Katz *et al.,* 1991).

The possible similarities between MCF and Burkitt's lymphoma had already been observed by Plowright in 1953. This was confirmed by molecular analysis which showed that certain clones derived from AHV-1 are similar to regions of the *Epstein-Barr virus* nucleotide sequence (EBV, responsible for the development of Burkitt's lymphoma) (Hsu *et al.,* 1990a; Katz, 1991). Molecular analysis showed that AHV-1 reference strains WC11 and C500 should be included in the gamma 2 group of the herpesviruses (*Rhadinoviruses*) whose other T-cell tropic members are *Herpesvirus saimiri* and *H. atalese* (Bridgen *et al.*, 1989). The genomes of these viruses consist of an unique region of circa 130 kbp of DNA flanked by two repeat regions summing to a total size of circa 160 kbp.

Further molecular analysis gave evidence of the relationship between herpesviruses isolated from different members of the subfamily Alcelaphinae. The two species of wildebeest (*C. taurinus, C. gnu*) were shown to be the natural hosts of AHV-1, whereas the hartebeest (*Alcephalus buselaphus*) and the topi (*Damaliscus lunatus*) were shown to be the host of a distinct virus, AHV-2 (Heuschele and Fletcher, 1984, Seal *et al.,* 1987, 1989, 1990).

1.2.2.2 *Ovine Herpesvirus-2* (OHV-2)

The epidemiological relationship between cattle which develop MCF and their earlier contact with sheep (Chapter 1.2.1.2.), has long been recognised by scientists working outside the African continent. On the principles of serological (Hamdy *et al.,* 1980; Rossiter, 1981, Herring *et al.,* 1988, Reid and Buxton, 1989, Schuller and Silber, 1990) and molecular analogies (Bridgen and Reid, 1991), a virus in sheep and SA-MCF affected cattle distinct from, but similar to AHV-1, has been identified, which is now classified as *Ovine herpesvirus-2* (OHV-2) (Roizman *et al.,* 1982).

Although the virus has not been isolated in the classical sense, it can be propagated in lymphoblastoid cell lines derived from diseased animals. Some established cell lines can transmit disease experimentally following intravenous inoculation into deer and

rabbits. (Berrie *et al.,* 1984; Reid *et al.,* 1985a; Cook and Splitter, 1988; Reid *et al.,* 1986; Schuller, 1990). From such a cell line, clones of the region of the OHV-2 viral DNA has been obtained which were homologous with regions of AHV-1 DNA (Bridgen and Reid, 1991). A fragment within one clone which did not hybridise with AHV-1 DNA was sequenced to provide an OHV-2 specific PCR which allows the amplification of genome from lymphoblastoid cell lines, affected cattle and normal sheep (Baxter *et al.,* 1993; Li *et al.,* 1995a,b). Therefore, in an unconventional way, the postulates of Koch have been confirmed.

1.2.2.3 *Hippotragine Herpesvirus-1* (HipHV-1) and other similar Herpesviruses

Serological surveys have shown that several species of wild ruminants belonging to the subfamilies Alcelaphinae and Hippotraginae in Africa are serologically positive for AHV-1, indicating that they harbour the same or similar viruses which potentially could be a source of MCF in cattle (Reid *et al.,* 1975; Hamblin and Hedger, 1984; Heuschele *et al.,* 1984). A serologically related virus, isolated from a roan antelope (*Hippotragus equinus*), induced MCF in rabbits following experimental inoculation (Reid and Bridgen, 1991) and was designated *Hippotragine Herpesvirus-1* (HipHV-1). Reid and Rowe (1978) isolated a similar herpesvirus from hartebeest (*Alcephalus buselaphus*), which was pathogenic for cattle and rabbits following experimental infection (Anon, 1973). Mushi and Karstadt (1981) isolated a similar virus from topi (*Damaliscus korrigum*), but suggested that it was not pathogenic for cattle.

Serological investigations (Parihar *et al.,* 1975; Heuschele *et al.,* 1984; Schuller and Silber, 1990) and the application of a diagnostic PCR for OHV-2 (Wiyono *et al.,* 1994) also suggested that domestic goats are infected with a similar virus, but no further data are yet available.

Since herpesviruses are normally species specific and co-evolve with their natural hosts as a fascinating mean of survival, it is probable that every single ruminant species has its own γ-herpesvirus. The pathogenicity of the different viruses for other species would have to be established in transmission experiments, but the

epidemiological data do not suggest that herpesviruses other than AHV-1 and OHV-2 are important sources of MCF in cattle or deer.

1.2.3 Clinico-pathological Features of MCF

The disease was first defined as a clinico-pathological entity and, even after the identification of the aetiological agents, this definition has been maintained. Experimental transmission and field observations of cattle, deer, buffalo and Bali cattle have shown, that it is not the differences in the causal agents which determine the pathological picture, rather it is the response of the specific host which varies markedly (Reid and Buxton, 1989).

The lesions found in experimentally infected rabbits are discussed in more detail in chapter 3.

1.2.3.1 Clinical Symptoms and Gross Pathology

The clinical signs and the gross lesions of MCF affecting the subfamily **Bovinae** have been most closely investigated. The disease is best known in domestic cattle (*Bos taurus*), water buffalo (*Bubalus bubalis*) and Bali cattle (*B. Javanicus*).

Goetze (1930), who described the disease in detail for the first time, distinguished four distinct clinico-pathological features of MCF in cattle still widely used today:

I. peracute generalised disease

II. intestinal disease

III. head-and eye disease

IV. mild disease

The **peracute disease** is characterised by sudden high fever followed in one to three days by death of the animal without other major clinical signs. Gross post mortem examination may reveal few obvious lesions. Peripheral lymph nodes are enlarged and oedematous with broad white cellular cortices. The spleen may be swollen. The **intestinal disease** is similar to the peracute form, but is of longer incubation and characterised by intense, sometimes haemorrhagic, diarrhoea. Peripheral lymph

nodes are enlarged and the intermandibular space can be oedematous. Catarrhal discharge can be observed from eyes and mouth and the animal dies almost invariably within 4-9 days. The gross lesions reflect the clinical picture with hyperplasia of the lymph nodes which can be sometimes haemorrhagic and/or necrotic and haemorrhagic enteritis. Furthermore, hepato-splenomegaly and erosive cystitis are often observed. Reddening and erosion of the buccal papillae are not uncommon if the course of the disease is relatively long. The **head and eye disease** generally lasts for one to three weeks. The main features are fever, initially serous discharge from the eyes, nose and mouth which later becomes catarrhal and purulent, progressive bilateral opacity of the cornea sometimes with erosion and ulceration, and hyperplasia of the peripheral lymph nodes (Figure 1.1). Photophobia is common. Nervous symptomatology such as depression, opisthotonos, circling and hyperaesthesia may be observed. Normally the animal does not survive. Gross pathological lesions can be relatively mild and non specific. The peripheral lymph nodes can be enlarged due to hyperplasia and oedema or smaller due to degenerative processes, but are always affected. Hepato-splenomegaly and cystitis are common, but not necessarily present. In the kidney, small white nodules are often present. Occasionally non suppurative arthropathy can be observed. Observations of the **mild disease** mostly relate to experimental transmission studies (Goetze, 1930) and is probably not recognised in the field. It is characterised by slight fever and hyperplasia of peripheral lymph nodes and the animal survives without any further complications.

Recently, **cutaneous MCF** was described (Holliman *et al.,* 1994; Michel, 1994a). The disease had a chronic course and was characterised by fever and exudative inflammation of the skin, especially on the neck, the back and the flanks which later resulted in multiple fissuring and secondary bacterial infection. On post mortem examination, the subcutaneous lymph nodes were enlarged and the skin changes were confirmed. No other gross lesions were found.

Examination of peripheral blood lymphocytes of animals with MCF showed leucopenia associated with relative monocytosis (Goetze, 1930; Plowright, 1953a; Pierson *et al.,* 1978).

In field cases, not all the clinical and pathological signs may be present and it is not always possible to categorise clearly the type of MCF. The only consistent symptoms are fever and involvement of the peripheral lymph nodes. Experimentally induced WA-MCF was more likely to induce head and eye form whereas experimentally induced SA-MCF predominantly caused the intestinal form of the disease (Pierson *et al.,* 1979). However, the authors have hypothesised that this variation could be due to the experimental conditions.

It is difficult to determine the **incubation period** under field conditions, since it is not always easy to establish the precise timing of infection. Pierson *et al.,*(1973) suggested that the incubation period in cattle could be up to 144 days in OHV-2 induced MCF. Experimentally, the incubation period varies depending on the inoculum and the route of infection. Plowright (1968) established a mean incubation period of 20 days after injecting cattle **intravenously** with AHV-1. Similar time periods were observed by others (Straver and van Bekkum, 1979; Kalunda *et al.,* 1981). Since animals were infected intravenously, it can be assumed that the incubation period following natural infection would be longer. In a **contact transmission** study, bovine calves exposed to viraemic wildebeest calves, developed the disease between 30 and 81 days after contact (Plowright, 1965b). Intra-nasal infection of cattle with AHV-1 induced the disease in a mean incubation period of 43 days (Mushi and Wafula, 1982)

In species of the family **Cervidae** (Chapter 1.2.1.4), MCF is considered to be the most serious viral disease in farmed deer (Beatson, 1985; Krogk *et al.,* 1987). Generally, it is the peracute form of MCF which is observed in these animals and if lesions develop, haemorrhagic enteritis is almost always present (Tong *et al.,* 1960; Huck *et al.,* 1961; Wyand *et al.,* 1971; Sanford, 1975; Reid *et al.,* 1979, 1987; Denholm and Westbury 1982; Oliver *et al.,* 1983).

1.2.3.2 Microscopic Lesions

Though the gross pathology of MCF is inconsistent, the microscopic lesions are always spectacular and are often not evident from the macroscopic examination. Furthermore, the histological changes are similar in cattle and other species of the family Bovinae and Cervinae, regardless of whether the disease is induced by AHV-1 or OHV-2. The histopathological changes (reviewed by Mettam 1923; Selman *et al.*, 1974; Liggitt *et al.*, 1978; Liggitt and De Martini, 1979, 1980 b, c; Reid and Buxton, 1989) can be summarised as follows:

I. hyperplasia of lymphoid organs which, in protracted cases, results in degeneration

II. interstitial accumulations of mononuclear lymphocytes adjacent to epithelial structures

III. paracheratotic hypercheratosis associated with lymphoid accumulations in the pregastric tract

IV. widespread segmental vasculitis

The **hyperplasia of lymphoid organs** can be classified in splenic and lymph node lesions. In the spleen, marked hyperplasia of the periarteriolar lymphoid sheaths (PALS) with relatively little follicle development is observed. When the disease is protracted, the spleen can be considerably depleted with only a few agglomerates of lymphoid cells left (Reid *et al.*, 1979). The hyperplasia of lymph nodes is due to expansion of the paracortex. Depending on the extent of alterations, necrosis and haemorrhages can be found in the cortex. The medulla of the lymph node is generally packed with macrophages and lymphoblasts. Follicles and germinal centres are lacking in the outer cortex (Selman *et al.*, 1974). Arteritis in the hilum is frequently present. Not all lymph nodes are affected to the same extent. Usually the most severely affected lymph nodes are those draining organs in which lymphoid cell accumulations are found (see below). Segmental, mononuclear cell **vasculitis** is a characteristic lesion (Daubney and Hudson, 1936; Berkman *et al.*, 1960). Most

commonly vasculitis (arterioles, veins and venules) is detected in the gastrointestinal tract, oral epithelium, eye, kidney, liver and lung. Vasculitis in the rete mirabilis is found to be pathognomonic (Liggitt and DeMartini, 1980b). Interstitial **accumulations of mononuclear lymphocytes** can be found next to biliary epithelium (liver and gall bladder), trachea, urinary bladder, various glands (pancreatic, lachrymal, salivary) and the cornea (Liggitt and DeMartini, 1980c). Focal **paracheratotic hypercheratosis** can be observed in the epithelia of the pregastric tract. The epithelial degeneration is always associated with lymphoid cell accumulations beneath the basal membrane and often ballooning degeneration can be observed. Granulocytes are few, particularly in mild lesions and the lamina propria is usually not affected (Liggitt and DeMartini, 1980c).

The mononuclear cells infiltrating all pathological lesions seem mainly to be lymphocytes, lymphoblasts with a few macrophages (Edington *et al.,* 1979; Liggitt and De Martini 1980 b, c; Patel and Edington 1980, 1981). More detailed characterisation of the lymphoid cell hyperplasia will be discussed in chapter 1.2.6.2.

1.2.4 Diagnosis

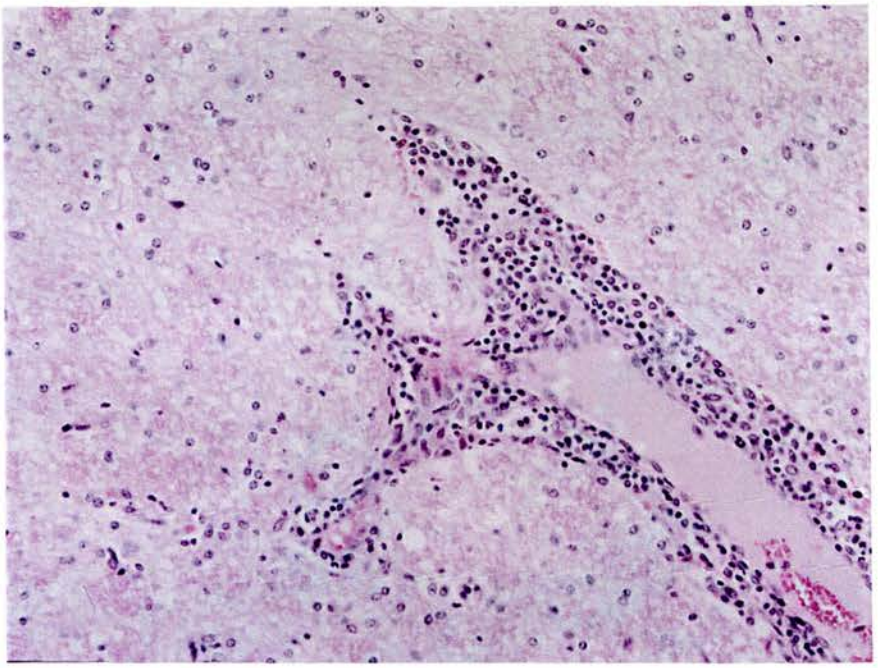
Since MCF has been defined as a pathological syndrome, definitive diagnosis has been based mainly on detection of histopathological changes. The problem with this is that the great variability or even absence of clinical signs makes clinical diagnosis difficult to impossible. With the identification of AHV-1 and more recently OHV-2, serological and molecular methods are now available which together with the clinical and pathological examination allow more precise diagnosis as well as permitting confirmation of infection in clinically affected animals.

1.2.4.1 Pathology

The clinico-pathological changes are the basis for the diagnosis of MCF. The histopathological changes (Chapter 1.2.3.1), which allow a definite diagnosis, are generalised segmental vasculitis, cuffing in the CNS (especially rete mirabilis) (Figure 1.2), hyperplasia of the lymph nodes and lymphoid cell accumulations in the kidney, urinary and gall bladder (Chapter 1.2.3.2). In Europe and North America, the

disease can often be confused with mucosal disease and bovine rhinotracheitis, whereas elsewhere Theileriosis, Trypanosomiasis, Rinderpest, Ibaraki and Jembrana Disease must be considered also (reviewed by Plowright, 1981; Reid and Buxton, 1989).

Figure 1. 2 Typical lymphoid cell cuffing in the central nervous system found in cases of MCF (case BJ 1104)



1.2.4.2 Virus isolation and Serology

Virus recovery and the demonstration of neutralising antibodies is as yet only possible for AHV-1. From clinical cases, virus can be recovered in tissue culture only from *fresh* tissues such as lymph node, buffy coat or spleen. Typical cytopathic effects, such as syncytia, intranuclear inclusion bodies and cell lysis can normally be observed within 1 to 3 weeks and the specificity of the cytopathic effect can be shown by immunofluoresence. The neutralising activity of sera from infected animals can be demonstrated using the cell-free reference strain WC11 (Plowright, 1965; Ferris *et al.,* 1976; Wibberely, 1976).

The diagnosis of WA-MCF in cattle can be confirmed also by serological investigation using indirect immunofluorescence (IIF). The diagnosis of SA-MCF is possible by the same method because of the antigenic relation between OHV-2 and AHV-1, although BHV-4 should be used as a control due to possible cross reactivity with this virus (Plowright, 1967; Reid *et al.,* 1975; Rossiter, 1980b; 1983; Rossiter and Jessett, 1980, Rossiter *et al.*, 1977, 1978, 1980). The serological diagnosis of SA-MCF was improved recently and a competitive-inhibition enzyme-linked immunosorbent assay (CI-ELISA), based on monoclonal antibodies to AHV-1 has been described (Li *et al.,* 1994a). Since all these assays are mainly based on the cross reactivity of AHV-1 and OHV-2, appropriate controls are necessary to avoid false positives due to other known bovine herpesviruses. Furthermore, not all affected animals develop antibodies due to the acuteness of the disease, the damage to the immune system or the chronic debilitating process of the disease (Plowright, 1967).

1.2.4.3 Polymerase Chain Reaction (PCR)

The molecular characterisation and sequences of AHV-1 allowed the development of a PCR which is a useful tool for the detection of aetiological agents present at very low concentration in the samples. Furthermore, it is very precise and relatively quick when compared to virus isolation. The PCR for detecting AHV-1 was developed by Hsu *et al.,* (1990b), Katz *et al.,* (1991), Michel (1993) and Tham *et al.,* (1994) whereas the PCR for OHV-2 detection was developed by Baxter *et al.* (1993).

1.2.5 Prevention

The control and prevention of MCF is based on the **separation** of the natural hosts and disease-susceptible animals especially during the peri-parturient period of the natural hosts (reviewed in Reid and Buxton, 1989).

As will be discussed in the chapter 1.2.6, high and persistent neutralising antibody titres are not protective against the disease, and, therefore, no classical **vaccine** has been developed yet (Piercy, 1954a; Reid and Rowe, 1973, Plowright *et al.,* 1975; Barnard 1984; Rossiter *et al.,* 1982a; Mirangi, 1991a, b).

1.2.6 Immunology and Immunopathology

The immune response to MCF-inducing viruses is complicated and not well understood. Acute infection induces the production of antibodies in the natural hosts as well as in the disease-susceptible species. However, after infection, the natural host remains infected for life as the immune response forces the virus to go into latency. On the other hand, antibodies do not seem to prevent disease in the disease susceptible host. In disease-susceptible species, primary infection induces a lymphoproliferative disease and only if it survives does an animal become resistant against disease.

Since virus isolation and antigen preparation is only possible for WA-MCF, most of the studies have been completed with AHV-1. However, the antigenic relationship between AHV-1 and OHV-2 permits speculation on the similarities between the two viruses.

1.2.6.1 The immune response in the natural host: induction of latency?

All adult individuals of the natural hosts are probably seropositive to their respective virus, but apparently do not develop any disease associated with infection (Chapter 1.2.1). The antibodies can be detected by virus neutralisation and IIF (Plowright, 1965; 1967 1968; Reid *et al.,* 1975, Rossiter, 1981). Most young animals acquire neutralising antibodies from the colostrum and all animals appear to become

seropositive in the first months after the decline of the maternal antibodies (Plowright, 1967; Li *et al.,* 1995b). It is believed, that, in these first months, primary infection occurs through contagion, though intra-uterine infection has been observed (Plowright, 1967). The primary replication site of AHV-1 appears to be the upper respiratory tract and the eye, since cell-free virus can be isolated from ocular and nasal secretion in young wildebeest. For a period of time, the virus can also be detected in the cell-associated form in blood. However, the virus can only be isolated from wildebeest for a limited time after acute infection (Mushi, 1980, Mushi *et al.* 1980 a, b, c; Plowright, 1965).

The acute infection with AHV-1 generates an active immune response characterised by the induction of secretory IgA in nasal secretions and by circulating virus-neutralising antibodies (Rurangirwa *et al.,* 1982; Plowright, 1967). However, since cell-associated viraemia is only temporary and herpesviruses characteristically spread by cell-to-cell contact, cell-mediated immune responses must be involved as well. AHV-1 is not cleared from the animals and can be reactivated in adult animals by immunosuppression or as a result of stress induced by pregnancy or transport (Rweyemanu *et al.,* 1974; Plowright 1965, 1972, 1986; Sanford *et al.,* 1977). These data indicate that AHV-1 establishes a latent infection in wildebeest. Latent infection, or latency, is defined as persistent infection in which viral transcription is minimised. In the case of α–Herpesvirinae, the genome can only be detected in nervous tissue and in the cornea and in lymphocytes in the case of γ-Herpesvirinae (Stevens, 1989). The exact mechanisms by which latency is induced are not known, but it is thought that immunological forces drive the virus to become latent and are responsible for maintaining this state. This strategy for survival, characteristic of all herpesviruses, has never been investigated rigorously for AHV-1 and to an even lesser extent for OHV-2. Recent developments in immunology and the availability of molecular techniques will hopefully reveal the sites of primary viral replication and latency.

1.2.6.2 The disease: death or survival!

Primary infection of disease-susceptible species results *almost* invariably in lethal lymphoproliferative disease after a relatively long incubation period (Chapter 1.2.3.). Such animals develop antibodies which can be detected by virus neutralisation (only AHV-1), IIF, complement fixation and CI-ELISA, though the titre can be variable (Plowright, 1967; Reid *et al.,* 1975; Hamdy *et al.,* 1980; reviewed by Rossiter, 1981; Li *et al.,* 1994a). It was shown for AHV-1 affected animals, that IgM and IgG appear simultaneously approximately one week before the onset of pyrexia (Rossiter, 1982). However, various studies in rabbits and cattle showed, that even high antibody titres are not protective against challenge with cell-associated virus regardless of the nature of antigen used for their induction (Plowright *et al.,* 1975, Barnard 1984, Rossiter *et al.,* 1989, Mirangi ,1991a,b).

That **protective immune status** can be induced is apparent from experiments in which animals recovered from AHV-1-induced MCF failed to develop the disease following rechallange. In these animals, virus was shown to persist in a latent form as off-spring of a recovered mother developed MCF shortly after birth (Plowright *et al.,* 1972). Furthermore, treatment with dexamethasone induced the recrudescence of MCF in an apparently recovered animal (Heuschele *et al.,* 1985). These findings strongly suggest that the major mechanism by which protection is induced involves mainly cell-mediated immunity similar to that observed in the natural host.

The presence of antibodies during disease together with the fact that vasculitis is a major pathological feature of MCF, led to the assumption that the pathogenesis might involve the deposit of immune complexes of viral antigen and antibody, similar to that observed in immune complex disease such as serum sickness, rheumatoid arthritis and some forms of glomerulonephritis (Roitt *et al.,* 1993, 21.1). Immunisation experiments, however, did not provide evidence to support a relationship between high antibody titres and reduction of the incubation period, while extensive studies failed to demonstrate immune complexes in tissues of affected cattle, rabbits or deer (Liggitt and DeMartini, 1980; Rossiter, 1980,1981; Patel and Edington, 1979; Mushi and Rurangirwa, 1981). A further reason to dismiss

this theory was based on the fact that immune complex disease is characterised by the presence of polymorphonucleated granulocytes, whereas MCF is distinguished by the presence of mononuclear lymphoid cells.

Identifying the **location of virus** in diseased animal has been the aim of several studies. Cell-associated virus and viral genome can be found in blood by virus isolation and PCR and consequently in almost all tissues during the disease, whereas cell-free virus has never been recovered from affected cattle or deer (Plowright *et al.,* 1975;. Mushi, 1980; Baxter *et al.,* 1993). These findings concur with the epidemiology of MCF that cell-free virus is not produced in the disease-susceptible species and thus they can not be the source of infection for other animals (Chapter 1.2.1). Furthermore, Piercy (1954) in his analysis of AHV-1 affected rabbits did not detect any inclusion bodies which indicate the presence of herpesvirus. Liggitt *et al.,* (1979, 1980) could not find viral particles, using electron microscopy. Using IIF, Edington *et al.,* (1979) and Patel *et al.,* (1981) failed to reveal many cells positive for viral proteins with rabbit antiserum to AHV-1 either in rabbits or cattle affected with the WA-MCF. Similarly, Bridgen *et al.,* (1992) did not find evidence of significant amounts of AHV-1 specific DNA by *in situ* hybridisation in MCF-affected rabbits. These studies indicate that in the lesions, only approximately one cell in 10^5 has detectable virus or viral product.

Viral expression increased after short term culture of single cell suspensions derived from lymph node and spleen from AHV-1 infected rabbits (Patel and Edington 1980, 1981), indicating that the virus is repressed *in vivo* by mechanisms involving cell-to-cell contact and/or systemic mediators. Study of a selection of explanted lymph node cells in the rabbit model showed that T-cells and macrophages retain infectivity (Mushi *et al.,* 1981, Mushi and Rurangirwa, 1981). It is in fact possible to establish **lymphoblastoid cell lines,** similar to HVS immortalised cell lines (Falk *et al.*, 1972; Abalshi *et al.*, 1985), from lymphoid organs of MCF-affected animals which harbour viral genome, but do not require antigen stimulation (Reid *et al.,* 1983; Berrie *et al.,* 1984). Cell lines were established using Con A blast derived supernatants (Cook and Splitter, 1988) and exogenous interleukin-2 (Reid *et al.,* 1989). All cell lines which

have been reported so far express T-cell surface antigens and are CD4, CD8 and γ/δ positive (Burrells *et al.,* 1990). Most of these cell lines are cytotoxic which suggests that they have the characteristics of lymphokine-activated killer cells (LAK-cells) (Cook and Splitter, 1988; Reid *et al.,* 1989). These data suggest that a severe dysregulation of the immune system is the principle pathogenic mechanism responsible for MCF. A further indication that the immune response to the viruses is altered is given by the observation that rabbits can develop cutaneous delayed hypersensitivity reaction to viral antigens during the incubation period, but that with the onset of the disease, this capability is abrogated (Mushi and Rurangirwa, 1981).

The involvement of **T-cells** *in vivo* was shown by Buxton *et al.,* (1984), who used polyclonal anti-rabbit T-cell serum to characterise the mononuclear lymphoid cell infiltrate in OHV-2 infected rabbits. However, the T-cell accumulations were inhibited by treatment with Cyclosporin A (Cs A) during the incubation period, although induction of pyrexia and degenerative lesions were not, indicating that other mechanisms must also be important for the development of MCF (Buxton *et al.,* 1984). The phenotypic analysis of the lymphoid cell accumulations in brains of MCF-affected cattle showed that mainly $CD8^+$ T-cells were present (Ellis *et al.,* 1992; Nakajiama *et al.,* 1992).

These results certainly suggest that the main target cell of the viruses of MCF are T-cells, in which viral transcription is limited *in vivo* and that it is likely that the virus induces its pathogenic potential through as yet unidentified secondary events involving these cells.

1.3. Interleukin-2 and its receptor system

The interaction of interleukin-2 (IL-2) with its receptor complex ***induces*** proliferation and differentiation of a number of leukocytes most importantly **antigen-specific T-cells** (Morgan *et al.,* 1976). Other functions of IL-2 are the activation of **B-cells** (Waldmann *et al.,* 1984; Zubler *et al.,* 1984; Ralph *et al.,* 1984; Mingari *et al.,* 1984; Saiki *et al.,* 1988), **NK-cells** (Henney *et al.,* 1981; Siegel *et al.,* 1987), **neutrophilic granulocytes** (Djeu *et al.,* 1993) and **macrophages** (Malkovsky *et al.,* 1987; Holter *et al.,* 1987).

IL-2 is mainly ***expressed*** by **T-cells** after antigen presentation or stimulation with various mitogens, but it has also been reported to be secreted by **B-cells** (Taira *et al.,* 1987; Walker *et al.,* 1988).

Recently, it has also been shown that IL-2 has effects in the **nervous system** as a neuromodulator (Hanisch *et al.,* 1993; Nistico and de Sarro 1991; Nistico 1993), as a growth factor for glial cells (Benviste and Merrill, 1986; Arzt *et al.,* 1993) and as a regenerative factor for axons (Eitan and Schwartz, 1993; Eitan *et al.,* 1994). The role of IL-2 in the central nervous system was underlined by the fact that mice transgenic for human IL-2 and IL-2R under the control of MHC class I ($H\text{-}2K^d$) had 40 - 70% less Purkinje cells in the cerebellum without the presence of inflammatory cells (Ishida *et al.,* 1989).

The biochemical properties of IL-2 are reviewed in chapter 7.

1.3.1 IL-2: a T-cell regulator

In resting T-cells, IL-2 is transcribed at a low level if at all (Leonard *et al.,* 1985). Once stimulated via the antigen receptor complex, the levels of IL-2 transcription increase together with the IL-2R expression, forming a positive loop which induces the **clonal expansion of antigen-specific T-cells** of all phenotypes (Meuer *et al.,* 1984; Kronke *et al.,* 1985; Depper *et al.,* 1985, Foxwell *et al.* 1990).

IL-2 is not only important in the multiplication of T-cells, but also in their **activation**. IL-2 co-induces the transcription of other cytokines, such as IL-4 and IFNγ *in vitro* (Ferrar *et al.,* 1986; Howard *et al.,* 1983) and *in vivo* (see below). In addition, IL-2 increased the ability of rat lymphocytes to migrate across vascular endothelium (Pankonin *et al.,* 1992).

Many studies have been carried out on the identification of T-cell subsets producing IL-2. Mosman *et al.,* (1986) subdivided mouse **T-helper cells** in the various subsets, namely $CD4^+$Th0, $CD4^+$Th1 and $CD4^+$Th2, by their cytokine profile. In this classification, Th0 clones (progenitor Th cell) and Th1 clones (involved mainly in cellular immunity) are able to produce IL-2 whereas Th2 clones (involved mainly in humoral immune response) do not produce it (reviewed by Annie *et al.,* 1990). This classification has been found useful in the investigation of various physiological immune responses in the mouse, but these Th subsets could not be differentiated on the basis of specific cell surface markers. Furthermore, it proved to be difficult to find these subsets in other species (reviewed by Kelso, 1995).

A similar classification has been attempted for $CD8^+$ T-cells. $CD28^+CD8^+$ T-cells have cytotoxic activity and produces IL-2 in response to activation signals, whereas $CD11b^+CD18^+CD8^+$ T-cells respond to IL-2 and are antigen specific suppresser cells (Carabasi *et al.,* 1991; Freedman *et al.,* 1991). The cytokine profile of human $CD8^+$ clones specific for *Mycobacterium leprea* showed that all cells expressed INFγ but could be divided into two subsets on the basis of their capacity to secrete IL-4, IL-5, IL-6 or GM-CSF. However, IL-2 was produced by both subsets (Salgame *et al.,*

1991; Kemeny *et al.,* 1994). A similar cytokine profile was found for murine CD8+ clones which all seem to be capable of expressing IL-2 (Annie *et al.,* 1990).

Administration of IL-2 to mixed populations of lymphocytes *in vitro*, can induce **lymphokine activated killer (LAK) cells**. These cells derive mainly from NK-cells, but it has been shown that other T-cell subsets can also be precursors of LAK-cells. The characteristic of these cells is their ability to be cytotoxic for a large variety of cells, mainly virus infected and tumour cells, unrestricted by MHC (Grimm *et al.,* 1982, 1983; Ramsdell and Golub, 1987; Grimm and Owenschaub, 1991).

Although antigen activation of resting T-cells is normally followed by induction of the IL-2 gene, complete activation is mediated by antigen specific receptor and through a co-stimulatory molecule. In the absence of a costimulatory event, the T-cell responds only partially and more importantly enters a state of unresponsiveness known as **clonal anergy** (Quill and Schwartz 1987; Jenkins and Schwartz, 1987; Arnold *et al.,* 1993). The characteristic of these anergic cells is their inability to produce IL-2 upon restimulation, although they continue to express IL-2R (reviewed by Schwartz, 1990, Ramsdell and Fowlkes, 1990). This extra-thymic mechanism for the maintenance of self tolerance and tolerance is thought to play a role in the pathogenesis of autoimmune disease.

The generation of alloreactive cytolytic T cells and resident NK cells after **administration of IL-2 to mice** showed the importance of the cytokine *in vivo* (Hefeneider *et al.,* 1983). High doses of IL-2 induced T-cell multiplication in lung, liver, spleen, kidney and mesenteric lymph node. Intermediate multiplication could be seen in blood and CNS, whereas the thymus, intestines, and skin were not affected (Ettinghausen *et al.,* 1985). Moreover the parental administration of IL-2 increased the serum levels of IFNγ and TNFα by induction of the expression of the corresponding genes in peripheral blood mononuclear cells (Kasid *et al.,* 1989; Hirano *et al.,* 1990; Sarvetnick *et al.,* 1990).

1.3.2 IL-2 receptor complex: α, β and γ subunits

The IL-2R complex is composed by three subunits: α, β, and γ (Waldmann, 1993).

The **IL-2Rα subunit** (CD25 or Tac antigen) was first detected by a monoclonal antibody (Uchiyama *et al.,* 1981) which identified this receptor on adult T-cell leukaemia tumour cells and on mitogen activated human T-cells. The receptor subunit is composed of a 55 kDa glycosylated, sulphated and phosphorylated protein. The transcriptional activation of the IL-2Rα gene is mediated through an enhancer element containing multiple regulatory sites, including NF-κB, CArG and Spl binding sites (Lowenthal *et al.,* 1989; Muegge and Durum, 1989). Transfection assays showed that the Tac antigen expression in non-lymphoid cells resulted in only low-affinity binding sites (Sabe *et al.,* 1984; Greene *et al.,* 1985), whereas in transfected lymphoid cells low and high affinity binding sites could be detected (Hatakeyama *et al.,* 1985; Wano *et al.,* 1987). Furthermore, it was shown that certain lymphoid cells respond to IL-2 in the absence of IL-2Rα or presence of anti-Tac (Trinchieri *et al.,* 1984; Ortaldo *et al.,* 1984; Ralph *et al.,* 1984). These findings led to the discovery of the **IL-2Rβ subunit**, a protein of 70-75 kDa (Tsudo *et al.,* 1986; Sharon *et al.,* 1986; Robb *et al.,* 1987; Teshigawara *et al.,* 1987). The IL-2Rβ chain is constitutively expressed on NK cells, monocytes and at least a subset of resting T-cells such as mature $CD8^+$cells. Recently, another subunit has been identified by mutation experiments (Imler and Zurawski, 1992; Zurawski and Zurawski, 1992). The co-expression of the various subunits alters the affinity for IL-2 binding and modulates the effect of IL-2 (Kuziel and Greene, 1991).

A defect of the human **IL-2Rγ chain** was described to be associated with a severe atypical X chromosome linked combined immunodeficiency (Noguchi *et al.,* 1993; Di Santo *et al.,* 1994). The disease is characterised by severely depressed cellular and humoral immunity. T-cells are absent and B-cells, while present, are abnormal in their function. These data suggest that the γ-chain was shared by more than one cytokine receptor (Leonard *et al.,* 1994). In fact, the IL-2Rγ chain was found to be a

functional component of IL-4 (B-cell stimulatory factor, BSF-1, or B-cell differentiation factor, BCDF-γ reviewed by Banchereau and Ryebak, 1994), IL-7 (important mediator of the B- and T-cell progenitors reviewed by Edington and Lotze, 1994) and IL-9 (T-cell and mast cell regulator reviewed by Renauld and Van Snick, 1994) (Kondo *et al.,* 1993 a, b; Noguchi *et al.,* 1993; Russell *et al.,* 1993).

Furthermore, the IL-2 receptor is important for the signal transduction of the newly described cytokine IL-15 (an NK-activation factor produced by monocytes/macrophages: Carson *et al.,* 1994). Using transgenic cells it was demonstrated that IL-15 used the β and γ chains of the IL-2R complex, but not the α chain (Giri *et al.,* 1994). This was confirmed by blocking the growth activity of initially IL-2 dependent ATL cell lines with monoclonal antibodies against the distinct subunits of the IL-2R. It was shown that another cytokine IL-T, possibly identical to IL-15, acts through the IL-2Rβ (Bamford *et al.,* 1994).

1.3.3 Transgenic mice

The study of transgenic mice made it possible to establish the role of IL-2 and IL-2R *in vivo* more closely, with the advantage (or disadvantage) of also altering the ontogeny of development.

Transgenic mice, carrying the **human IL-2 gene** under the control of the MHC class I promoter ($H2K^d$), showed high serum levels of IL-2, weight loss, apparently irreversible alopecia, pneumonia and most died within a year (Ishida *et al.,* 1989a). Transgenic mice for IL-2 and IL-2R (Tac) under the same promoter died within 4 weeks from pneumonia. The infiltrating cells observed were NK-cells, indicating that *in vivo* NK-cells are the main target cell for IL-2 (Ishida *et al.,* 1989b). These data also suggested that IL-2 is not indispensable for the development of the thymus-dependent cells.

Mice transgenic for the **human IL-2Rα** under the control of SV40 promoter had a degenerate thymus, an increase in double negative T-precursor cells and loss of double positive thymocytes, indicating that IL-2Rα is required for normal growth and

differentiation of T-lymphocytes (Gutierrez-Ramos *et al.,* 1989). The expected autoimmune disease was not observed in either of these transgenics.

More information on the role of IL-2 *in vivo* has been obtained by inactivation of the endogenous IL-2 gene in mice. Surprisingly the **knockout** mice, produced by targeted recombination of the IL-2 gene, had increased serum concentration of IgG_1 , but the thymocytes matured and mature circulating T-cells developed which proliferated in response to Con A though feebly (Schorle *et al.,* 1991). The *in vivo* immune response to virus infection of these IL-2 deficient mice seemed to be in the normal range, but if they survived for more than 3 months they developed an ulcerative-colitis like disease with an autoimmune pathogenesis (Sadlack *et al.,* 1993, Kundig *et al.*, 1993). In knockout mice for IL-2 and Il-4, the proliferation of T-cells seemed increased if examined by *in vivo* incorporation of bromo-deoxyuridine even though the proportion of the single cell types were maintained (Sadlack *et al.,* 1994). This shows that IL-4 can partially compensate for the IL-2 deficiency. In contrast, IL-2 deficient mice with a BALB/c genetic background develop, in approximately 5 weeks, a generalised autoimmune disease characterised by haemolytic anaemia, hyperplasia of lymphoid tissue and mononuclear inflammatory reactions in pancreas, liver, lung and mediastinum histologically similar to the changes observed in MCF (Sadlack *et al.,* 1995). These authors hypothesised that the one possible role of IL-2 *in vivo* might be the maintenance of self tolerance. These findings confirm the existence of alternative pathways of T-cell maturation and proliferation. Whereas children with IL-2-deficiency have severe combined immunodeficiency, indicating the importance of IL-2 *in vivo*, they have a relatively normal number of T-cells (Weinberg and Parkman, 1990; Chatila *et al.,* 1990, Conley, 1992)

The evaluation of the role of IL-2 *in vivo* shows the complicated connection within one cytokine/cytokine receptor system, in as much that in distinct experimental situations different target cells were evaluated by using specific promoters. Overall, these data emphasise that IL-2 is more than a elementary T-cell growth factor.

1.4. Herpesviruses: Hijacking the Immune System

To 'survive', viruses must exploit normal cellular functions and elude the host immune system. **Herpesviruses,** such as *Herpesvirus saimiri* (HVS) (Fleckenstein and Desrossiers, 1982), *Epstein-Barr virus* (EBV) (Masucci and Ernberg, 1994), *Human herpesvirus-6* (HHV-6) (Dewhurst, 1994), *Human herpesvirus-7* (Dewhurst, 1994; Abalashi *et al.*, 1995), *Murine herpesvirus-68* (MHV-68) (Nash and Sunil-Chandra, 1994, Efstathiuo *et al.*, 1990) and *Equine herpesvirus-2* (EHV-2) (Agius and Studdert, 1994), have elaborated this to elegant perfection. The relation of OHV-2 and AHV-1 to this group of viruses is based on sequence analysis, epidemiological similarities and their lymphotropisms (Table 1.2).

The short review will illustrate the variety of strategies by which these viruses influence the immune system. The first part will concentrate on the transforming activity of HVS, the most closely related virus to AHV-1 and OHV-2, illustrating the immunological consequences of latent infection. The second part will focus on viral homologues to host products which are implicated in the regulation of the immune system.

Table 1. 3 Comparison between viruses of the MCF complex and other Herpesviruses (for references see 1.2 and 1.4)

Feature	MCF complex	HVS	EBV
Herpesvirus (HV)	γ-HV group 2	γ-HV group 2	γ-HV group 1
Lymphoproliferative Disease	mononuclear lymphoid cells	T-cell lymphomas	B- and T-cell lymphomas
Transmission	inter- and intraspecies	inter- and intraspecies	intraspecies
Latency	yes	yes	yes
Disease in Natural Host	no	no	yes
Viral Expression in the Lesion	low	low	low
Cell-free Virus in the Lesion	no	no	no
Antibodies Protect against Infection/Disease	no	no	no
Cell Lines from the diseased individuals	T-cell (CD4, CD8, γ/δ)	T-cell (CD4, CD8)	mainly B-cell, but also T-cell
IL-2-dependence of T-cell lines	yes	most	yes
Pathogenesis	?	oncogenes cytokine upregulation	oncogenes, virokine, cytokine dysregulation,

1.4.1 Transforming activity of *Herpesvirus saimiri*

Squirrel monkeys (*Saimiri sciureus*) are the natural host of ***Herpesvirus saimiri*** (HVS), a γ-herpesvirus which induces lymphoproliferative disease in various species of New World monkeys (*Callithrix*, *Saguinus*). It is not possible to detect cell-free virus in the disease-affected animals and viral expression can be found only in a few T-lymphocytes. Immortalised T-cell lines, which do not permit the complete replicative cycle of the virus, can be established from the lymphoid organs of these monkeys, similar to the B- and T-cell lines established with EBV (review of HVS in monkeys: Fleckenstein and Desrossiers, 1982; genomic structure of HVS: Albrecht *et al.*, 1992; review for biology of EBV: Purtilo *et al.*, 1992). The transformation is mainly due to **oncogenes** situated in the left terminal part of the unique region of the viral genome. Most of these genes have homology with genes of EBV. Two transforming proteins, unique to HVS have been characterised, namely Stp-C (saimiri transformation associated protein of C strains) and Tip (tyrosine kinase interacting protein) (review of transformation by HVS: Meinl *et al.*, 1995).

Certain strains of HVS can **immortalise human T-cell clones** (Biesinger *et al.*, 1992). Even though these cell lines become independent of antigen-stimulation, they maintain their antigen-specificity (Weber *et al.*, 1993). The immortalised cell lines maintain the cell surface markers of their original clones (CD2, CD3, CD25, CD11a/CD18, CD58, CD8 or CD4) (CD4: DeCarli *et al.*, 1993; CD8: Berend *et al.*, 1993). The growth of these initially IL-2 dependent cell lines is density dependent, indicating that **cell surface antigens** are important to maintain the cell growth. The autocrine growth of the transformed cell lines is mediated by **CD2** and its ligand CD58, since the multiplication can be blocked by the respective monoclonal antibodies whereas cross linking of the two surface receptors has a stimulatory effect (Del Prete *et al.*, 1994; Mittrückner *et al.*, 1992). Antibodies to the IL-2R complex and CD4 reduced the IL-2-dependent proliferation (Broker *et al.*, 1994).

Transformation with HVS lowers the threshold of CD2 mediated **cytotoxicity**, enhances lectin-dependent cytotoxicity of Th1 cells and enables Th2 cells to become cytolytic (Mittrückner *et al.,* 1993; Berend *et al.,* 1993).

The transformed cell lines constitutively express their original **cytokine** pattern (CD4 Th1 or Th2). Additionally, TNFα and IL-3 production is upregulated, which might be costimulatory to the autocrine growth (De Carli *et al.,* 1993). Since cytokines are produced constitutively, or even enhanced, and the growth of these cell lines is Cyclosporin A sensitive (Chou *et al.,* 1995), the cell-associated virus interacts with **promoters for a variety of immunologically active proteins**. The relevance of all these events *in vivo* has still to be evaluated, but it becomes evident from these data that the latent virus interferes significantly with the regulation of the immune system, inducing the expression of cytokines.

1.4.2 Virokines and other viral homologues of cellular proteins

Apart from the transforming activity of HVS, herpesviruses possess a variety of homologues of immunologically important proteins.

Sequence analysis and subsequent expression revealed that the open reading frame BCRF-1 of EBV codes for a bioactive **IL-10** homologue, which promotes B-cell growth and blocks IL-2 and IFNγ expression in stimulated human PBMCs. Viral IL-10 is expressed during the lytic phase (Hsu *et al.,* 1990; Stewart *et al.,* 1994). An IL-10 homologue has also been identified in the genome of EHV-2 (Rode *et al.,* 1993, 1994). HVS encodes for an homologue of a new cytokine, **IL-17** or CTLA-8, which costimulates T-cell proliferation (Rouvier *et al.,* 1993, Yao *et al.,* 1995)

Chemokines are a class of structurally related, chemoattractant cytokines involved in the recruitment mainly of monocytes and neutrophils, acting through the receptor of IL-8 and MIP-1/RANTES (Review: Murphy, 1994; Ahuja *et al.,* 1994). Viral homologues for chemokine receptors have been found in both HVS and human cytomegalovirus respectively (Ahuja *et al.,* 1993; Neote *et al.,* 1993; Gao and Murphy, 1994). The recent analysis of the genome of *human herpesvirus-6* (HHV-

6), EHV-2 and other lymphotropic herpesviruses revealed coding sequences similar to that of cellular chemokines themselves and regions with motifs related to chemokine receptors (also called G-protein coupled serpentine receptor) (Gompels *et al.,* 1995; Nicholas *et al.,* 1992).

Other proteins of HVS have strong relationships to proteins involved in **cell division**. The best characterised are the viral homologues of the cellular type D cyclin, and two proteins involved in DNA synthesis (thymidylate synthetase and dihydrofolate reductase) (Albrecht *et al.,* 1992; Jung *et al.,* 1994). Another example of potential viral interference with the defence system of the host, is the presence of an HVS-homologue of **CD59** (protectin), a regulatory protein of the complement cascade which protects blood cells, leukocytes and some epithelial cells from complement-mediated lysis (Rother *et al.,* 1994).

Homologues to host sequence motifs, important for the regulation of the immune system have been recognised. The **AUUUA repeat** occurs at the 3' end non coding regions of cytokine genes and the same motif is involved in the post-transcriptional regulation promoting the degradation of the messenger RNA. Four small RNAs (HSUR-1 to 4) of HVS possess the same motif and it is thought that the resulting competition might influence the pathogenesis of the disease (Myre *et al.,* 1992, Chou *et al.,* 1995; Geck *et al.,* 1994 a, b). The cytoplasmic domains of the **transducing subunits associated with B and T cell antigen receptors** contain a common amino acid motif (Tyr-X-X-Leu/Ile) important for the activation of these cells. It was shown that the latent membrane protein 2A (LMP-2A) of EBV contains the same motif. Data derived from site directed mutagenesis studies suggest that oligomerization of LMP-2A might trigger the activation and proliferation of infected B-cells and contribute to viral persistence (Beaufils *et al.,* 1993).

Although many of these results are based on sequence analysis and the biological activity of the viral homologues has still to be demonstrated, the data suggest that molecular mimicry of host proteins is an important pathogenic mechanism used by lymphotropic herpesviruses.

1.5. Aim of the Study

Malignant catarrhal fever is a pathological syndrome characterised by the presence of mononuclear lymphoid cells in almost all tissues and the lack of readily demonstrable viral antigens. The pathology is therefore not caused by the destruction of particular cells as seen in other viral infections, but involves unconventional pathogenic mechanisms. The many similarities between the viruses of the MCF-complex (AHV-1, OHV-2, HipHV-1) and related herpesviruses, led to the hypothesis that MCF might be induced by a dysregulation of the cytokine cascade (Table 1.2). At the beginning of this study, IL-2 was considered to be the most likely candidate responsible for hyperplasia since its function is to activate and thus to promote the proliferation of T and NK-cells. The hypothesis that the lymphoid hyperplasia was caused by a hyperproduction of IL-2 was therefore tested.

The second objective of this study was the phenotypic and functional characterisation of the mononuclear lymphocytes involved in the disease process.

For the investigation of these aspects of the disease, two models were used. The AHV-1 infected rabbit model was employed to study *in vivo* aspects of MCF (Chapters 3 to 7), whereas OHV-2 positive bovine cell lines derived from clinical cases of MCF were used to evaluate the biological consequences of OHV-2 latency (Chapters 8 and 9).

Chapter 2

Materials and Methods

2.1 Animals

2.1.1 Rabbits

Conventionally reared male and female New Zealand rabbits 3-12 months old were used for all experiments. Rabbits were inoculated intravenously with pathogenic AHV-1, OHV-2 or HipHV-1 (Chapter 2.1.3), rectal temperatures were monitored every day and animals were killed on the second day of febrile disease ($\geq 40^0$C). For control purposes, rabbits were either not inoculated, inoculated with non pathogenic derivatives of AHV-1 (Chapter 2.1.3) or immunised with different antigens intramuscularly with incomplete Freund's adjuvant (Chapter 2.1.3). Animals were killed by anaesthesia using a fluothane/CO_2 mixture and tissues collected for histological examination or preparation of single cell suspensions (Chapter 2.4.6).

2.1.2 Cattle

Clinical cases of MCF in cattle referred by the Scottish Agricultural College Veterinary Services and practising veterinary surgeons were transported to the Moredun Research Institute. After clinical examination, animals were killed by injection with pentobarbital (Euthetal, Rhone Mereux, UK), exsanguinated, necropsied and various tissues taken for histological examination. In addition, spleen, retropharyngeal, prescapular, prefemoral and mesenteric lymph nodes were collected aseptically and single cell suspensions were prepared (Chapter 2.4.6).

2.1.3 Inoculum

Pathogenic inoculum Rabbits were inoculated intravenously with 1 ml of inoculum consisting of approximately 5 x 10^7 spleen cells derived from rabbits reacting with MCF which corresponds to approximately 5 times the tissue culture infectious dose per 50% ($TCID_{50}$). This dose had been established in previous experiments in this institute as 10 $^{2.9}$ $TCID_{50}$ 10^6 splenocytes (Bridgen *et al.*, 1992). The inoculum was stored in cryoprotectant (Chapter 2.4.4) in liquid nitrogen. The viral strains used for the various experiments are summarised in table 2.1. The viruses used were maintained by serial passage in rabbits, since they could not be maintained in cell culture (Chapter 1.2.2).

Table 2. 1 Pathogenic strains used to induce MCF in rabbits

Virus	*Strain*	*Reference*
AHV-1	C 500	Plowright *et al.*, 1960
European OHV-2	86/13	Reid *et al.*, 1986
Indonesian OHV-2	BJ 873; BJ 880	Wiyono, unpublished
HipHV-1	BJ 668	Reid and Bridgen, 1991

Non-pathogenic inoculum The *in vitro* passages of the strain C500 result in loss of pathogenicity (Handley *et al.* 1995). Rabbits were infected with 1 ml of approximately 5 x 10^7 spleen cells derived from a rabbit inoculated with the cell cultured passaged C500. The animals were maintained for at least three months, were clear of clinical signs and the absence of histological changes was confirmed.

Hyperimmunised animals These animals were used to distinguish a 'physiological' immune lymphoid hyperplasia from the malign hyperplasia observed in MCF. The animals had received CsCl pelleted rotavirus or recombinant proteins from Mycobacterium paratuberculosis (p40). The course of hyperimmunisation was given by a series of intramuscular injections of the antigen resuspended in incomplete Freund's adjuvant with the last injection given between two weeks and six weeks prior to the post mortem.

2.2 Pathological Examination

The required organs were cut in blocks of appropriate size and fixed in 10% formalin saline for the appropriate period of time. The tissues were processed by standard methods for histopathological examination or by St. Marie processing for immunocytochemistry. After the processing, tissue sections of 4 μm were prepared which were stained with eosin and heamatoxylin for histological examination or used for immunocytochemistry (Chapter 2.3.1)

2.2.1 Standard processing

Tissue blocks were processed on a Shandon Hypercenter XP processor with the following program:

Step	Reagent	Time	Temperature
1	90% ethanol	45 min	20^0 C
2	98% ethanol	60 min	20^0 C
3	98% ethanol	60 min	20^0 C
4	98% ethanol	75 min	20^0 C
5	98% ethanol	75 min	20^0 C
6	isopropanol	90 min	20^0 C
7	isopropanol	120 min	20^0 C
8	isopropanol:toluene (1:1)	90 min	20^0 C
9	toluene	60 min	20^0 C
10	toluene	60 min	20^0 C
11	paraffin	90 min	60^0 C
12	paraffin	90 min	60^0 C

2.2.2 St. Marie processing

The whole procedure was performed at 4^0C. Tissues were fixed in 10% formalin saline for 3-4 hours and then transferred to 95% ethanol overnight. The tissue blocks were dehydrated further with 98% ethanol (two times for 30 min) and absolute ethanol (two times for 30 min). Thereafter the blocks were rinsed in absolute ethanol/xylene (2:1) for 10 min and in xylene/absolute ethanol (2:1) for another 10 min. Then the material was washed three times in xylene for 20 min. Xylene

was then replaced by paraffin using a vacuum embedder for four cycles of 10 min. Tissues were embedded in paraffin and blocks stored at 4^0 C.

2.3 Immunocytochemistry

2.3.1 Immunohistology

Sections of 4 µm were cut from the required tissue blocks, mounted on VECTABOND treated slides and dried overnight at room temperature. The sections were dewaxed, if required, by rinsing them in xylene and ethanol. Endogenous peroxidase was blocked by placing the sections for 30 min in 1% hydrogen peroxidase in methanol. The following procedures were performed in a humid chamber. After washing twice for 5 min with washing buffer (26 mM sodium phosphate, 155 mM sodium chloride, pH adjusted to 7.5, 0.45% Tween 80), non-specific staining was blocked by applying normal goat serum (inactivated at 65^0 C for 60 min, diluted 1:4) (Vector, UK) for 30 min. Excess serum was blotted using filter paper and sections incubated overnight at 4^0 C with the relevant mouse monoclonal antibody diluted appropriately in high salt buffer (0.5 M NaCl, 26 mM sodium phosphate). The slides were washed twice for 10 min in washing buffer and incubated for 30 min with the secondary antibody (biotinylated goat-anti-mouse in washing buffer, 1:800) (Vector, UK). After two further washes, the sections were incubated with the anti-biotin-complex (Vectastain Elite ABC-Kit, Vector, UK) for a further 30 min. Sections were washed in wash buffer two times for 15 min and the reaction visualised with di-amino-benzidine and H_2O_2. Sections were washed under running tap water and counterstained with Meyer's haematoxylin. After further washes in water as required, sections were dehydrated in graded ethanol, cleared in xylene and mounted using a xylene-based mountant.

2.3.2 Flow Cytometry (FACScan)

Single cell suspensions (Chapter 2.4.2) were prepared at a concentration of 5 x 10^6 cells/ml in FACS medium (1% FBS, 500 IU/ml heparin (Sigma, UK), 0.1% sodium azide in PBS). The procedure was performed on ice in V-bottomed 96 well plates. To 50 µl of cell suspension, 50 µl of the required primary antibody diluted in FACS medium was added and incubated for 90 min. The cells were centrifuged at 150 g and the supernatant removed. The cells were washed twice with FACS medium and incubated with fluorescein isothiocynate (FITC) conjugated antibody (sheep-anti-mouse 1:50) (Dakopatts, Denmark) for 30 min. The cells were washed twice more, fixed in 1% paraformaldehyde and transferred to FACS-tubes (Falcon 2054, Becton Dickinson). Analysis was performed with a Becton Dickson Facsan (Mountain View, CA) with linear amplification for forward (FSC) and side scatter (SSC) and logarithmic amplification for FITC green fluorescence (FL-1). Lymphocytes were distinguished on the basis of the FSC/SSC profile.

2.4 Cell culture

2.4.1 Wash Medium (WM)

The wash medium (WM) was made up with balanced Hank's medium supplemented with 2% foetal bovine serum (FBS), 100 IU/ml penicillin, 50 ug/ml streptomycin (Northumbria Biologicals, UK) and 500 U/ml heparin (Sigma, UK).

2.4.2 Cell Culture medium (CCM)

The cell culture medium (CCM), routinely used was Iscove's modification of Dulbecco's medium (IMDM) (Gibco, UK) supplemented with 10% FBS, 100 IU/ml penicillin, 50 ug/ml streptomycin, 2 IU/ml fungizone (Roussel, UK) and 50 µM 2-mercaptoethanol.

2.4.3 Culture conditions

Cells were incubated in 10 ml flasks (Corning Inc., Corning, New York), 24-well cell culture plates or flat bottomed 96-well cell culture plates (Nunclon TM, Intermed, Denmark) in humidified atmosphere at 37^0 C with 5% CO_2.

2.4.4 Cryopreservation

If necessary the cells were stored at -70^{0}C in liquid nitrogen in CCM supplemented with 40% FBS and 10% dimethyl sulfoxide (Sigma, UK).

2.4.5 Supplements

The interleukin-2 (IL-2) used if not otherwise stated was human recombinant IL-2 (Eurocetus, UK). The recombinant bovine IL-2 (Collins et al. 1994) was kindly provided by Dr. R.A. Collins from the Institute for Animal Health (Compton, UK), Concanavalin A (Con A) was purchased from Difco laboratories (Detroit, USA) and was usually used at a concentration of 5 μg/ml. The Cyclosporin A (Cs A) used in the cell culture experiments was purchased from Sandoz (Sandimmun, Switzerland).

2.4.6 Preparation of single cell suspensions

The lymphoid tissue was removed by aseptic technique. The tissue was finely chopped with scissors and put through a stainless steel sieve. The cell suspension was then washed with WM, resuspended in the same medium, layered onto LYMPHOPREP (Nycomed, Oslo, Norway) in equal volumes and centrifuged at 500 g for 30 min. Whole blood collected in vacutainers containing preservative-free heparin was layered directly on LYMPHOPREP and treated in the same manner. The interface was collected, washed three times with WM and counted using a haematocytometer before resuspending the cells at the desired cell concentration in culture medium.

2.4.7 Determination of cell multiplication by incorporation of thymidine

Samples of cells were seeded in triplicate at the appropriate cell concentration in flat bottomed 96-well cell culture plates. The cells were pulsed with 0.5 μCi/well tritiated thymidine in a volume of 50 μl for 20 hours prior to harvesting them onto glass fibre filters (Packard Instruments, The Netherlands). The cell associated radioactivity was analysed using the β- scintillation counter MATRIX 96 and each

well was counted for three minutes. The final counts were expressed in mean counts per minute (cpm).

2.5 Molecular Biological Analysis

2.5.1 RNA extraction

The RNA extraction was performed following the technique of Chomczynski and Sacchi (1987).

The cells used for the experiments were centrifuged at 150 g to obtain a pellet of 1 x 10^6 cells, dissolved in 1 ml RNA extraction buffer (Chapter 2.6) and stored at -70^0 C. The previously prepared cell extracts were thawed and 0.5 ml aliquots transferred to Eppendorf tubes. 50 µl 3 M sodium acetate (pH 4.5) was added, followed by 0.5 ml water saturated phenol (Chapter 2.6). Finally 0.1 ml of chloroform was added and the mixture shaken vigorously for 5 min. The preparations were incubated on ice for 10 min. and then centrifuged on a microfuge for 10 min. The aqueous phase was transferred to fresh centrifuge tubes and an equal volume of isopropanol was added. After mixing well, the preparations were left at -20^0 C for at least two hours and then centrifuged for 20 min. on a microfuge. The pellets were washed in 70% ethanol, dried and redissolved in 1% di-ethyl pyrocarbonate (DEPC, RNase inhibitor) treated water.

2.5.2 Northern gel

To verify the presence of RNA, the samples were run on a Northern gel. The samples were incubated at 65^0C for 10 min in the presence of RNA loading buffer (Chapter 2.7). 2 µl of each sample was diluted in 8 µl of loading buffer and run on a 1% agarose/MOPS (Chapter 2.6) gel containing 2% v/v formaldehyde. The running buffer was 1X MOPS (Chapter 2.6). The gel was run for 45 min at 100 V and the RNA visualised with a UV-transilluminator.

2.5.3 Quantification of RNA

The concentration of RNA preparation was assessed by the measurement of its OD at 260 nm, using a spectrophotometer. An OD_{260} of 1 corresponds to 40 µg/ml total RNA. RNA preparations free from protein possessed an OD_{260}:OD_{280} ratio of approximately 2.0. The concentrations were verified by comparing the relative intensity of the ribosomal RNA bands from each sample.

2.5.4 Reverse transcription

The reverse transcription (RT) was performed in a total volume of 20 µl per reaction. The reaction buffer was composed of 1X RT-buffer (Boehringer, Mannheim), 0.2 µM dATP, 0.2 µM dCTP, 0.2 µM dGTP and 0.2 µM dTTP. To every reaction 4 U reverse transcriptase AMV (Boehringer, Mannheim) was added. If not otherwise stated, 0.125 A_{260} U/ml random primers (pd(N)6, Pharmacia, UK) were used for every reaction. The preparation was incubated at 37^0 C for 90 min. The cDNA was then diluted 1:5 in water, aliquoted and stored at -20^0 C.

2.5.5 DNA extraction

For the extraction of DNA for the diagnosis of MCF, single cell suspensions derived from buffy coat, lymph node, or spleen were used. 1 x 10^6 to 1 x 10^7 cells were suspended in 600 µl of DNA extraction buffer and mixed thoroughly. An equal volume of phenol (Chapter 2.6) : chloroform (6/4) was added and vortexed for 2 min. After 10 min of centrifugation on the microfuge, the aqueous layer was removed and mixed with half the volume of 7.5M ammonium acetate. The DNA was then precipitated with 100% ethanol at -20^0 for at least one hour.

2.5.6 Polymerase-chain reaction (PCR)

The reaction was carried out in 10 µM 3'end primer, 10 µM 5'end primer, 1X PCR-buffer (Boehringer, Mannheim), 0.2 µM dATP, 0.2 µM dCTP, 0.2 µM dGTP and 0.2 µM dTTP. For every reaction, 1 U Taq polymerase (Boehringer, Mannheim) was added. The PCR was performed in a total volume of 20 µl. 10 µl of previously

prepared cDNA was added to every reaction. Approximately 50 µl of sterile mineral oil was layered on the top. The mixture was incubated in a HYBAID OMNIGENE thermocyler. Products were then visualised by agarose gel electrophoresis. Unless otherwise specified the following program was used:

1. cycle:	5 min at 94^0 C	45 sec at 45 ^{0}C	and	45 sec at 72 ^{0}C
2. to 30. cycle:	60 sec at 94^0 C	45 sec at 45 ^{0}C	and	45 sec at 72 ^{0}C
31. cycle:	60 sec at 94^0 C	45 sec at 45 ^{0}C	and	8 min at 72 ^{0}C

2.5.7 Agarose Gel electrophoresis of DNA

To establish their size, the PCR products obtained were electrophoresed on 1% (w/v) Nucleic acid grade "ultrapure" agarose (BRL, UK) in 1X TBE gel (Chapter 2.6). Two volumes of DNA loading buffer were added to the samples prior to loading, and electrophoresis was carried out at 1-4 volts/cm in 1X TBE (Chapter 2.6) until the required resolution was achieved. Size estimation was achieved by co-electrophoresis of a 1 kbp ladder (Boehringer Mannheim, UK) and DNA was visualised under UV illumination.

2.5.8 Southern Blot

Transfer of PCR-products from agarose gels to nylon membranes was performed using a modified method of Smith and Summer (1980).

The agarose gel was incubated in denaturing solution (1.5 M NaCl, 0.5 M NaOH) twice for 15 min, washed in distilled water and incubated two times for 30 min in neutralising solution (1 M ammonium acetate, 0.02 M NaOH). The transfer onto Hybond-N (Amersham, UK) was carried out by placing the membrane on top of the gel together with layers of soaked and dry filter paper. The DNA was transferred to the membrane for at least 1 hour by capillary action. After drying the membrane, the DNA was fixed by UV-irradiation (0.4 J/cm) for 60-70 sec. The membrane could be used directly or stored in the dark at room temperature. The membrane was incubated with prehybridisation solution to block non-specific binding of the probe

for at least 30 min at 55^0 C in a hybridisation oven. After this the membrane was incubated overnight with the appropriate digoxygenin-labelled oligonucleotide (1.0 pmole/ml in prehybridisation solution) at the appropriate temperature. The product was visualised using the DIG Nucleic Acid Detection Kit (Boehringer, Mannheim), following the manufacturers protocol using the anti-digoxygenin alkaline phosphatase conjugate at a dilution of 1: 5,000.

2.6 Frequently used reagents

10X MOPS/EDTA:	0.2 M MOPS (3-N-morpholino-propano-sulfonic acid), 50 mM sodium acetate, 10 mM EDTA, adjusted to pH 7.0 and autoclaved.
1X TBE:	0.1 M TRIS, 0.1 M ortho-boric acid, 1 mM EDTA, 0.2 mg/ml ethidium bromide
20X SSC:	3 M NaCl, 0.3 M sodium citrate, adjusted to pH 7.0 with HCl
DNA extraction buffer:	150 mM NaCl; 10 mM Tris-HCl, pH 8.0; 100 mM EDTA and 0.1% SDS v/v
DNA loading buffer:	0.25% (w/v) bromophenol blue, 40 % (w/v) sucrose in 1X TBE
PBS:	137 mM NaCl, 26.8 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4
Phenol for DNA:	saturated with 10 mM TRIS-HCl, pH 8.0, 1mM EDTA (P 4557, Sigma, UK)
Phenol for RNA:	saturated with distilled water and 0.1% (w/v) B-hydroxyquinolin,
Prehybridisation solution:	5X SSC, 0.5% (w/v) Boehringer blocking reagent, 0.1% (w/v) N- laurylsarcosine sodium salt and 0.02% (w/v) sodium dedocyl sulphate (Bohringer, UK)
RNA extraction buffer:	4 M guanidine isothiocyanate (GIT), 50 mM TRIS-HCl pH 7.6, 10 mM EDTA, 2 % sodium lauryl sarkosinate, 142 mM β-mercaptoethanol
RNA loading buffer:	0.75 ml deionsed formamide, 0.15 ml 10X MOPS, 0.24 ml formaldehyde, 0.1 ml DEPC (1% in water), 0.1 ml 10% (w/v) bromophenol blue

Chapter 3

Pathology of rabbits experimentally infected with AHV-1, HipHV-1, European and Indonesian OHV-2

3.1 Introduction

It was established in early studies of MCF, that the wildebeest-associated form of the disease could be transmitted to rabbits using live cell suspensions from affected cattle (Daubney and Hudson, 1936). Plowright (1953 a, b) described in detail the pathological lesions in rabbits infected experimentally with AHV-1 and concluded that the lesions resembled the pathological findings in cattle with WA-MCF. The main feature was hyperplasia of lymphoid organs with the destruction of small lymphocytes followed by production in large number of medium and large lymphocytes, many of which were undifferentiated cells. Piercy *et al.,* (1955) analysed the pathology induced by AHV-1 in 300 rabbits. The lymph nodes were described as generally enlarged, congested and often haemorrhagic and necrotic. Such changes were seen most frequently in the precrural, inguinal and submandibular lymph nodes. The spleen was hyperplastic and lymphoid cell accumulations were observed in the kidney and liver.

Pattison (1946) claimed to have transmitted MCF to rabbits from fatal cases of head and eye forms of bovine MCF in Palestine, but provided no further details about the epidemiology. However, transmission of the sheep-associated form of the disease, induced by OHV-2, was achieved from affected red deer (*Cervus elaphus*) in the UK (Buxton and Reid, 1980). Subsequently, Westbury and Denholm (1982) transmitted MCF to rabbits from affected Rusa deer (*C. timorensis*) in Australia and Oliver *et al.,* (1984) in New Zealand transmitted the disease from affected red deer. The transmission from SA-MCF-affected cattle to rabbits was later achieved by Reid *et al.,* (1986). The main pathological feature in rabbits with SA-MCF was described by the above mentioned authors as hyperplasia of spleen and lymph nodes.

Reid and Buxton (1989) suggested a different pathological pattern in AHV-1 and OHV-2 infected rabbits. They observed, that peripheral lymph nodes such as popliteal and submandibular lymph nodes were significantly larger in AHV-1 than in OHV-2 infected rabbits. In contrast, mesenteric lymph node and GALT were more hyperplastic in OHV-2 infected rabbits than in AHV-1 infected rabbits. The spleen

was consistently involved in both infections, though in the AHV-1 induced form of the disease, splenomegaly seemed to be more pronounced.

In the present study, the investigation of MCF in rabbits has been extended to OHV-2 derived from Indonesian and HipHV-1. Gross as well as histological lesions were evaluated to establish possible differences between the disease induced by the various viruses.

3.2 Materials and Methods

Fourteen rabbits were infected with one of the four isolates of bovid γ-Herpesvirinae. All were monitored clinically and killed on the second day after the onset of the fever (Chapter 2.1.1). Three rabbits were inoculated with pathogenic AHV-1, three rabbits with European OHV-2, three rabbits with Indonesian OHV-2 and 5 rabbits with HipHV-1 (Chapter 2.1.3). Three animals were not inoculated and used as controls. The post mortem examination was performed and gross lesions were recorded. Spleen, appendix, mesenteric, popliteal and submandibular lymph nodes as well as various non lymphoid organs (trachea, lung, oesophagus, liver, kidney, lachrymal gland) were removed. From these tissues, histological sections were prepared and stained with haematoxylin and eosin (H&E) (Chapter 2.2.1). The changes were recorded and scored subjectively. Samples from the three control rabbits were similarly processed.

3.3 Results

All infected rabbits became febrile with a mean incubation period of 15.2 days (SEM 2.07). No difference in incubation period between the groups could be detected. The animals were generally depressed and stopped eating and drinking. Hyperplasia of the lymphoid organs and accumulations of mononuclear lymphoid cells were the main feature of the experimentally induced disease. The control animals showed no obvious lesions. The pathological changes observed in the different lymphoid and non lymphoid organs are summarised in the tables 3.1 to 3.7.

Spleen The organ was greatly enlarged (up to two/three times normal), dark in colour and the pulp was engorged and soft. Expansion of the periarteriolar lymphoid sheaths was detected histologically and , in the red pulp, single heterophilic polymorphonucleated cells (hPMN) were present to a variable degree. There was no obvious significant differences between the four infected groups (Table 3.1).

Table 3. 1 *Splenic pathology* in rabbits experimentally infected with AHV-1, HipHV-1 Indonesian and European OHV-2

Animal	*splenomegaly*	*PALS* [1]	*hPMN*
Indon. OHV-2			
AS 92/3	+ [2]	+[2]	+++
AS 92/6	+	++	-
AS 92/8	++	+	-
Eur. OHV-2			
AS 92/12	++	++	-
AS 92/13	++	++	-
AS 92/14	++	++	+
AHV-1			
AS 92/4	+	+	++
AS 92/5	++	+	-
AS 92/7	++	++	-
HipHV-1			
AS 92/1	+	+	++
AS 92/2	+	+	++
AS 92/9	+	+	+
AS 92/10	+	++	-
AS 92/11	+	++	-

[1] periarteriolar lymphoid sheath; [2] grade of hyperplasia

Lymph nodes In the different groups, distinct lymph nodes were variably affected (Table 3.2). In HipHV-1 infected rabbits, the severity of the lesions observed in mesenteric, popliteal and submandibular lymph nodes was similar. In rabbits infected with European and Indonesian OHV-2, the mesenteric lymph node was more profoundly affected than the peripheral lymph nodes, whereas in the rabbits infected with AHV-1, the same type of lesion was found in the submandibular and the popliteal lymph nodes while the mesenteric lymph node was relatively unaffected.

The different distribution pattern between OHV-2, AHV-1 and HipHV-1 was statistically significant (Kruskal-Wallis Test: $p < 0.05$).

The affected lymph nodes were hyperplastic, altered in shape, haemorrhagic and oedematous with focal necrosis. Expansion of the paracortex was the predominant histological finding. Large amounts of mononuclear leukocytes were accumulating in the medullae and, in some cases, these cells were found in the capsule. Haemorrhages, oedema and necrosis were confirmed at histological examination.

Table 3. 2 *Lymph node pathology* in rabbits experimentally infected with AHV-1, HipHV-1, European and Indonesian OHV-2

Animal	*mesenteric lymph node*	*submandibular lymph node*	*popliteal lymph node*
Indon. OHV-2			
AS 92/3	+++	-	-
AS 92/6	+++	-	-
AS 92/8	+++++ N[1], H[2], S[3]	-	+
Eur. OHV-2			
AS 92/12	++++ S	-	+
AS 92/13	++++ H, S	+	+
AS 92/14	+++ H, S	-	+
AHV-1			
AS 92/4	-	+++ H	+++ H
AS 92/5	-	+++ H	+++ H
AS 92/7	-	+++ H	+++ H
HipHV-1			
AS 92/1	+++	+ H	-
AS 92/2	++	+ H	-
AS 92/9	+++++ N, H, S	+ H	+ H
AS 92/10	+++++ N, H, S	+ H	+ H
AS 92/11	+++ N, H, S	++ H	++ H

[1] necrosis; [2] haemorrhages ;[3] alteration of the shape;

Appendix The organ was enlarged in all infected rabbits. Macroscopic foci of necrosis, upto 1 cm in diameter, were observed in five rabbits (Table 3.3). Additionally, the sacculus rotundus and Peyer's patches were hyperplastic in HipHV-1 and OHV-2 infected rabbits.

Microscopically the lesions could be classified as:

I. lymphoid hyperplasia (distance from basal membrane to mucosal surface)

II. necrosis (variable grades of destruction of the mucosa)

III. apoptosis (death of single cells in B- and T-cell areas)

IV. presence of high number of hPMN and microabscesses

V. vasculitis in the submucosal structures (accumulation of lymphoid cells specifically around the blood vessels of the submucosa)

The T-cell areas were hyperplastic to a variable degree in all infected rabbits. In the group of AHV-1 infected rabbits, no significant lesions could be identified apart from a slight increase in size, some cell exudate on the mucosal surface and a consistent increase in the number of lymphoid cells in the submucosa. In rabbits infected with HipHV-1, Indonesian and European OHV-2, the mucosa was markedly hyperplastic. In these rabbits, cell death varying from apoptosis to complete necrosis of the T- and B-cell area was observed. Lymphoid cells were present in the submucosa and frequently spread to the muscular layer and the adventitia. The number of hPMN was particularly increased in the appendix of rabbits infected with HipHV-1 (Table 3.4). The difference between AHV-1 infected animals and animals infected with other viruses was statistically significant (Kruskal-Wallis Test: $p < 0.05$).

Table 3. 3 *Appendix pathology* in rabbits experimentally infected with AHV-1, HipHV-1, European and Indonesian OHV-2

Animal	*H*[1]	*N*[2]	*A*[3]	*hPMN*[4]	*vasculitis*	*MC*[5]
Indon. OHV-2						
AS 92/3	+	+/-	+++	+	-	-
AS 92/6	++	-	-	-	-	-
AS 92/8	++	+++++	+++	-	-	+++[6] N
Eur. OHV-2						
AS 92/12	+	-	-	-	-	++
AS 92/13	++	+++++	++	+++	+++	++ N
AS 92/14	++	+++++	+	+	+++	++
AHV-1						
AS 92/4	+	+/-	-	-	-	+
AS 92/5	-	-	-	-	-	+
AS 92/7	-	-	-	-	-	++
HipHV-1						
AS 92/1	+	+/-	-	+ MA	-	++
AS 92/2	++	-	-	+ MA	-	+++ N
AS 92/9	++	+++++	+++	+++	+	+++ N
AS 92/10	+	-	-	+	-	+++
AS 92/11	+	+++	+	+	+	+++ N

[1] lymphoid hyperplasia; [2] necrosis; [3] apoptosis; [4] infiltration of heterophilic polymorphonucleated granulocytes; [5] macroscopic changes; [6] increase in size

Trachea Congestion and haemorrhages of the tracheal mucosa was detected in all rabbits, infected as well as the control rabbits. It was most obvious in the thoracic part of the trachea. The lesion was therefore not considered to be a direct consequence of the experimental infection. The haemorrhages were confirmed microscopically. Submucosal accumulations of lymphoid cells were detected in two rabbits infected with AHV-1, in 2/5 rabbits infected with HipHV-1 and in 1/3 rabbit infected with Indonesian OHV-2. There was no statistically significant difference between the groups of infected animals.

Lung The most frequent macroscopic lesions were white, shiny, superficial nodules on the pleural surface. All rabbits infected with European OHV-2, four rabbits infected with HipHV-1, and 1/3 of the rabbits infected with AHV-1 showed this pathological change. Small haemorrhagic lesions on the surface as well as in the parenchyma were present in 1/3 rabbit infected with European OHV-2, 1/3 rabbit infected with AHV-1 and three of the rabbits infected with HipHV-1. The differences were not statistically significant.

Histopathologically the lesions, which were present independently of each other, were:

I. histiocytic response (accumulation of non-identifiable cells of variable size and number, which probably coincided with the white nodules found at the post-mortem examination)

II. vasculitis and more precisely phlebitis (similar to the lesion in IL-2 treated mice described by Ettinghausen *et al.,* 1985)

III. lymphoid cell accumulation around terminal bronchi and hyperplasia of MALT (secondary follicles)

IV. presence of hPMN

V. bronchiolitis (cell exudate in the lumen of the bronchiole)

The histiocytic response was present in all infected rabbits. The other responses were variable apart from preibronchial lymphoid cell accumulations in 3/3 AHV-1-infected rabbits (Table 3.4).

Table 3. 4 *Lung pathology* in rabbits experimentally infected with AHV-1, HipHV-1, European and Indonesian OHV-2

Animal	*HR*[1]	*MALT*[2]	*LA*[3]	*vasculitis*	*hPMN*[4]	*bronchiolitis*	*MC*[5]
Indon. OHV-2							
AS 92/3	++	+/-	+/-	+	+	-	
AS 92/6	+++	-	-	+	+++	-	
AS 92/8	++	-	-	-	-	-	
Eur. OHV-2							
AS 92/12	++	++	-	-	-	+	WN[6]
AS 92/13	+	-	-	-	+	+	WN
AS 92/14	++	-	-	-	-	-	WN, H[7]
AHV-1							
AS 92/4	+/-	+++	++	-	-	-	WN
AS 92/5	++	+++	++	+	+/-	-	
AS 92/7	+++	-	++	+	-	-	H
HipHV-1							
AS 92/1	+++	+	+++	+++	-	-	WN
AS 92/2	++	+++	++	-	+	+	WN, H
AS 92/9	+/-	+	-	+/-	+	+	WN
AS 92/10	++	+/-	-	-	-	+	WN, H
AS 92/11	++	+/-	-	-	-	-	H

[1] histiocytic response; [2] hyperplasia of MALT; [3] peribronchiolar lymphoid cell accumulations; [4] heterophilic polymorphonucleated granulocytes; [5] macroscopic changes; [6] white nodules observed on the surface of the lung; [7] haemorrhages

Oesophagus Macroscopic examination did not show any alteration, therefore the histological data are only presented for ten rabbits. At least one section was examined from every rabbit. Lesions were characterised by focal accumulation of lymphoid cells (mononuclear leukocytes and hPMN) in the submucosa. In what was probably an advanced stage of the pathological change, the basal membrane was interrupted and lymphoid cells seemed to invade the epithelium itself. The overlying epithelial cells showed ballooning. The lesion was not always present in every group.

To investigate this further, a set of 27 sections was prepared at random from the same rabbit (infected with Indonesian OHV-2). In four of the sections, no alteration was detected. In two of the sections, four foci of marked lymphoid infiltration and epithelial damage were present. In all other sections, at least one focus of lymphoid infiltration was present although the degree of infiltration was variable. This investigation revealed, that in the same rabbit the grade of alteration varies enormously and that a negative finding was not significant. Consequently it must be assumed, that this lesion could be present in all infected rabbits.

Liver White lesions of irregular shape could be detected macroscopically on the surface of the liver and on its cut surface. These were probably foci of coagulative necrosis which was found on microscopic investigation. This pathological change was present in 2/3 rabbits infected with European OHV-2, 1/3 infected with AHV-1, and 1/5 infected with HipHV-1 (Table 3.5). In two rabbits (HipHV-1, AHV-1) white shiny spots, 1-2 mm in diameter, were detected. The organ was friable in all infected animals.

The histopathological changes identified were:

I. peribiliary accumulation of mononuclear leukocytes

II. perivascular accumulation of mononuclear leukocytes

III. hPMN in the parenchyma, in the lymphoid accumulations and the presence of microabscesses

IV. fatty degeneration

V. congestion

Fatty degeneration as well as congestion were observed in all infected rabbits. Peribiliar lymphoid accumulations were present in all infected rabbits but one. The lesions observed in rabbits infected with OHV-2 were less frequent and the number of accumulating cells was lower. Perivascular accumulations were not present in all rabbits, even if the peribiliar accumulations were prominent. hPMN and microabscesses were observed in many rabbits (Table 3.6). There was no significant difference between the groups of infected animals.

Table 3. 5 *Liver pathology* in rabbits experimentally infected with AHV-1, HipHV-1, European and Indonesian OHV-2

Animal	*vLA*[1]	*bLA*[2]	*ahPMN*[3]	*ihPMN*[4]	*MC*[5]
Indon. OHV-2					
AS 92/3	+	++	-	+++++	-
AS 92/6	++	+++	++	+++	-
AS 92/8	++	+++	-	+	-
Eur. OHV-2					
AS 92/12	-	-/+	+	-	-
AS 92/13	+/-	++	-	+	CN[6]
AS 92/14	-	-	-	-	CN
AHV-1					
AS 92/4	++++	+++++	-	-	Nod[7]
AS 92/5	+	+++	-	-	-
AS 92/7	-	++++	-	++	CN
HipHV-1					
AS 92/1	+++	++	+++	+++	Nod
AS 92/2	-	+++	++	+++	-
AS 92/9	+/-	++	-	-	-
AS 92/10	-	+/-	+	++	-
AS 92/11	-	+	-	+++	CN

[1] perivascular lymphoid cell accumulations; [2] peribiliar lymphoid cell accumulations; [3] microabscesses; [4] infiltration of heterophilic polymorphonucleated granulocytes; [5] macroscopic changes; [6] coagulative necrosis; [7] white nodules on the surface of the organs

Kidney White spots on the surface of the kidney, which were probably due to fibrosis unrelated to the experimental infection were noted in two rabbits. No macroscopic lesions could be identified in any other rabbit.

Marked focal accumulations of mononuclear leukocytes associated with blood vessels in the cortex and the medullae could be identified in all rabbits infected with AHV-1. The same lesion was found in one rabbit infected with Indonesian OHV-2 and in 2/5 rabbits infected with HipHV-1. In one of the rabbits the lymphoid cell accumulations were clearly associated with the epithelium of the renal pelvis (Table 3.6). The difference between the OHV-2, AHV-1 and HipHV-1 infected rabbits was statistically significant (Kruskal-Wallis Test: $p < 0.05$).

Table 3. 6 *Kidney pathology* in rabbits experimentally infected with AHV-1, HipHV-1, European and Indonesian OHV-2

Animal	*vLA*[1]	*eLA*[2]
Indon. OHV-2		
AS 92/3	-	-
AS 92/6	-	-
AS 92/8	+	-
Eur. OHV-2		
AS 92/12	-	-
AS 92/13	-	-
AS 92/14	-	-
AHV-1		
AS 92/4	+++++	-
AS 92/5	+++	++
AS 92/7	+++	-
HipHV-1		
AS 92/1	+++++	-
AS 92/2	-	-
AS 92/9	-	-
AS 92/10	+++	-
AS 92/11	-	-

[1] perivascular lymphoid cell accumulations; [2] periepithelial lymphoid cell accumulations

Lachrymal Gland A white lachrymal discharge (250 µl) was observed in all rabbits a few minutes after death. The phenomenon was considered an artefact perhaps due to the anaesthesia. Mononuclear lymphoid cell accumulations were found histologically and, since variable numbers of leukocytes and plasma cells were present in all rabbits, only large numbers of these cells in an unusual location were considered of pathological significance. The lymphoid cell accumulations were noted around the excretory ducts and next to the blood vessels in all rabbits infected with AHV-1 and in 2/5 rabbits infected with HipHV-1. The rabbits infected with AHV-1 presented additionally peri-acinous lymphoid accumulations and in some cases complete substitution of single lobules with leukocytes.

Central Nervous System Vasculitis, perivascular cuffs or gliosis characteristic lesions in bovine MCF were not detected in any rabbit.

Vasculitis The widespread vasculitis, which is typical of the disease in cattle could not be consistently detected and very often could not be distinguished from the lymphoid cell accumulations (liver, kidney, lachrymal gland). In the following table (3.7), these dubious cases are also included. Only a few vessels, mainly veins, were damaged per section and the severity was never comparable to the lesions found in bovine MCF.

Table 3. 7 *Vasculitis* in rabbits experimentally infected with AHV-1, HipHV-1, European and Indonesian OHV-2

Isolate	*Organ*	*Number of affected animals/ total animals*
AHV-1	Trachea	1 / 3
	Lung	3 / 3
	Liver	2 / 3
	Lachrymal gland	2 / 3
	Kidney	3 / 3
HipHV-1	Lung	2 / 5
	Liver	2 / 5
	Lachrymal gland	1 / 5
	Kidney	2 / 5
	Appendix	2 / 5
Indonesian OHV-2	Lung	2 / 3
	Liver	3 / 3
European OHV-2	Appendix	2 / 3
	Liver	1 / 3

3.4 Discussion

3.4.1 The rabbit: an animal model for MCF

The characteristic pathological features found in this study were hyperplasia of the lymphoid organs (spleen, lymph nodes, MALT) and interstitial accumulations of mononuclear leukocytes next to epithelial structures such as biliary ducts, trachea, oesophagus and lachrymal glands. These findings confirm previous investigations by Plowright (1953b), Piercy *et al.,* (1955), Edington *et al.,* (1979), Patel and Edington (1980) and Buxton *et al.,* (1980). The histological examination of spleen and lymph nodes showed that most of the hyperplasia is probably due to the expansion of the PALS and the paracortex respectively. The vasculitis and brain lesions, so important for the diagnosis of the disease in cattle, was not reproduced consistently. These findings strengthen the hypothesis of various authors (Buxton *et al.,* 1984; Reid *et al.,* 1985, Reid and Buxton, 1989), that the cells which are mainly involved in the pathogenesis are T-cell. The typical lesions found in the pregastric tract of MCF-affected cattle (Chapter 1.2.3) are focal lymphoid cell accumulations at the basal membrane of the mucosa and paracheratotic hypercheratosis. The examination of the oesophagus of infected rabbits revealed the same kind of lesion and could therefore be used as a model for this specific pathological alteration. Since the presence of hPMN seems to be correlated with the grade of pathology found in the different organs, it can be suggested, that their appearance is secondary to the initial pathology.

Experimental infection of rabbits with HVS induces a lymphoproliferative disease which is similar to those observed in this study though lymphomatouse changes tend to be more pronounced (reviewed by Fleckenstein and Desrossiers, 1982). The disease is characterised by a terminal leucocytosis and the animals die 17 - 196 days post infection. Histopathological examination shows massive infiltration of most organs (brain included) with lymphocytic or lymphoblastic elements. The focus of these pathological descriptions of HVS-infected rabbits is the neoplastic process since most of the experiments were done in the seventies. The lymphoproliferation observed may therefore have been evaluated with different criteria. Degenerative

changes, as observed in AHV-1, OHV-2 and HipHV-1 infected rabbits, might also have been overlooked. Generally, it can be presumed that the pathological changes in HVS-infected rabbits and rabbits infected with AHV-1, OHV-2 and HipHV-1 are comparable. This underlines further the close relationship of this group of γ-herpesviruses (Chapter 1.4.).

Leporid herpesvirus-1 (LHV-1 or *H. sylvilagus*) and *-2* (LPV-2 or *H. cuniculi*) are the recognised herpesviruses which were isolated from cottontail rabbits and domestic rabbits respectively (Roizman, 1992). LHV-1 is a γ-herpesvirus which was shown to induce chronic infection with persistent low grade viraemia and experimental infection, can induce a lymphoproliferative disease, but only in rabbits of the genus *Sylvilagus* (Hesselton *et al.*, 1988). LPV-1 clearly possesses the capacity to produce disease in the laboratory rabbit and experimental infection can cause mononuclear leukocyte infiltration in the site of infection. Eosinophilic intranuclear inclusions in the corneal epithelium, the interstitial cells of the testis and in endothelial cells are characteristically found (Maré, 1974). Since the rabbits used in the present experiments were of the genus *Oryctolagus* and no inclusion bodies could be observed, both the leporid herpesviruses can be excluded as aetiological agents in the lymphoproliferative disease observed in the present study.

3.4.2 Different distribution patterns of lesions caused by AHV-1, OHV-2 and HipHV-1

In many studies (Chapter 1.2.6), the lymphotropism of MCFV has been demonstrated. This study shows that AHV-1, HipHV-1, European and Indonesian OHV-2 all affect the lymphoid system in the rabbit, but cause a **different distribution of lesions** depending on the virus responsible. The spleen, as an important systemic lymphoid organ, was involved in all cases. The lesions caused by the OHV-2 isolates from Indonesia and from UK were indistinguishable. OHV-2 infected rabbits had more severe lesions in the gut-associated lymphoid tissues (mesenteric lymph nodes, appendix), whereas rabbits infected with AHV-1 had more severe lesions in kidney, lachrymal gland, popliteal and submandibular lymph nodes.

In contrast, the HipHV-1 infected rabbits had lesions in both compartments: peripheral and gut-associated lymphoid tissues. The suggestion made by Reid and Buxton (1989), that the distribution pattern of lesions varies depending on the inducing virus (AHV-1, OHV-2), could therefore be confirmed.

The lack of readily demonstrable viral expression in the lesion led to the proposal of an indirect pathogenesis (Chapter 1.2.6.2). To investigate what induces the lymphoid hyperplasia in MCF, it is therefore necessary to further characterise the mononuclear lymphocytes as well as the lymphocyte populations in the lymphoid organs. Since it is not possible to distinguish the involved lymphocytes on simple histological basis, the cells were labelled with monoclonal antibodies (Chapter 4).

3.5 Summary

From the histological examination of rabbits infected with AHV-1, HipHV-1, European and Indonesian OHV-2, it can be concluded, that:

1. all four viruses induce MCF-like syndromes in the rabbit.
2. hyperplasia of the T-cell areas was reproduced with all four viruses.
3. European and Indonesian OHV-2 can be considered pathogens with a similar lymphotropism.
4. Lesions in the oesophagus of rabbits can be used as a model for the lesions found in the forestomachs in bovine MCF.

Chapter 4

Characterisation of leukocytes in MCF-affected rabbits

4.1 Introduction

Eukaryotic cells express a large number of different molecules (receptors for hormones and cytokines, adhesion molecules, etc.) on their surface which are important for their function. Many of these molecules have now been identified, mainly in mouse and human, with the use of monoclonal antibodies (mAb). This method of characterisation has been widely applied to studies of the immune system, where it is not possible to distinguish histologically between lymphocytes with different functions. An internationally recognised system was established for the characterisation of leukocytes and APC which is based on clusters of differentiation (CD). This system permits the division of lymphocytes not only into B- and T-cells, but into further phenotypic sub-populations. T-cells are characterised by the presence of CD3 and T-cell antigen receptor (TCR) involved in antigen recognition in the context of the major histocompatibility complex (MHC) (Roitt *et al.* 1993). Since mAb against CD3 of some species are not available, CD5 is used as an alternative T-cell marker. This antigen is present on almost 95% of T-cells, but its function has not yet been clearly identified (Ledbetter *et al.* 1981). Recently it has been shown, that CD5 is also present on a proportion of normal B-cells (Plate *et al.* 1993; Lydyard *et al.* 1993). Another marker which is often used to help in the characterisation of T-cells is CD43. This antigen is present on all leukocytes apart from mature B-cells, but differences in glycosylation allows identification of various subsets (Manjanath *et al.* 1995; Sperling *et al.* 1995; Rosenstein *et al.* 1993). The mAb L11/135 against rabbit CD43 used in this study is restricted to thymocytes and T-cells (Wilkinson *et al.,* 1992).

T-cells are further subdivided on the basis of the presence of CD4 and CD8 antigens which are co-receptors for CD3 and consequently involved in cell-bound antigen recognition (reviewed by Julius *et al.* 1993). A large majority of mature T-cells can be classified in $CD4^+CD8^-$ or $CD4^-CD8^+$ cells. The two subsets are functionally distinct. $CD4^+$ cells are MHC class II restricted helper cells, whereas $CD8^+$cells are MHC class I restricted cytotoxic cells. Both subsets have a precise role in modulating the physiological immune response in a specific direction.

As discussed in the previous chapters, the typical lesion of MCF is characterised by hyperplasia of T-cell areas in lymphoid organs, the presence of mononuclear lymphocytes in various non-lymphoid organs and the absence of readily demonstrable viral expression (Chapter 1.2.6.2 and 3). The identification of the T-cell subsets involved in this hyperplasia would therefore assist in the development of an hypothesis as to which regulatory mechanism(s) could be involved in the pathogenesis of MCF.

Since the model for WA-MCF is the best established, most of the work presented was performed using AHV-1 infected rabbits on the second day of clinical response. Hyperimmunised rabbits were employed as controls to demonstrate the difference between a physiological and pathological hyperplasia of the lymphoid system. Additionally, tissue sections derived from the animals infected with AHV-1, OHV-2 and HipHV-1 used in chapter 3 were examined by immunohistochemistry.

The characterisation of leukocytes in non-lymphoid tissues was performed immunohistochemically on tissue sections whereas the characterisation of leukocytes from spleen and popliteal lymph nodes was performed by flow cytometry (FACScan) on cell suspensions. The relatively few reagents available for immunological studies in rabbit and the difficulty in performing *in situ* labelling with some of the monoclonal antibodies, made FACS-analysis the most appropriate technique for the characterisation of cells in lymphoid tissues. Furthermore, the technique gives quantitative results which are more amenable to statistical analysis. The characterisation of non-lymphoid tissues was limited by difficulty in extracting enough cells from these organs. Double labelling in tissue sections and FACS analysis was not possible because of the small volume of mAb available.

4.2 Materials and Methods

4.2.1 Monoclonal Antibodies

Cells were characterised with mAb against CD43 (T-cells), CD5 (T-cells and a subset of B-cells), CD4 (thymocytes and helper/inducer T-cell subset), CD8 (thymocytes and cytotoxic/suppresser T-cell subset), NRBM (B-cell), CD25 (IL-2Rα unit) and sheep anti-IgG (B-cells, PMN and macrophages) (Table 4.1).

In addition, the mAb (PC10) against proliferating cell nuclear antigen (PCNA) which functions as a co-factor for DNA polymerase delta (Linden *et al.* 1992) and is mainly present during S phase of the cell cycle (Bolton *et al.* 1992) was used to investigate if lymphocytes in non-lymphoid tissues are in DNA synthesis and by implication, proliferating (Table 4.1).

Table 4. 1 Antibodies specific for rabbit antigens

Specificity	mAb	dilution used	source	Ref.
CD43	L11/135	1 : 1000	ascites[1]	Jackson *et al.*, (1983)
CD 5	Ken 5	1 : 5	S/N[3]	Kotani *et al.*, (1993)
CD 4	Ken 4	1 : 200	ascites[1]	Kotani *et al.*, (1993)
CD 8	12.C7	1 : 100	ascites[1]	De Smet *et al.*, (1983) Wilkinson *et al.*, (1992)
CD25 (IL-2Rα)	Kei α1	different dilutions	ascites[1]	Kotani *et al.*, (1993)
B-cell	NRBM	1 : 200	ascites[1]	Wilkinson and Gorton (1992)
anti-IgG		1 : 200	antisera[2]	
anti-PCNA	PC10	1 : 200	S/N[3]	Linden *et al.*, (1992)

1 mouse mAb; **2** developed in sheep; **3** cell culture supernatant

The mAb L11/135, Ken 4, 12C.7 and NRBM were kindly provided by Dr. Wilkinson from the Royal College of Surgeons, London. The mAb Ken 5 was purchased from Serotec, anti-PCNA from DAKO (Denmark) and anti-IgG from Sigma ImmunoChemicals (UK).

The optimal concentrations of these antibodies for use in FACScan were determined by testing the mAb on a rabbit-derived IL-2 dependent T-cell lines BJ 1006 as well as using different dilutions on cells from control animals (Chapter 4.2.3). Since problems arose with the use of the rabbit anti-CD25, the antibody was also tested on splenocytes derived from a control rabbit (Chapter 4.2.3). These cells were labelled immediately after the tissue was processed and after 48 hours in culture in the presence of 5 µg/ml Con A. The working dilutions for immunocytochemistry were determined by staining sections from control rabbits with a series of dilutions.

4.2.2 Immunohistochemistry

For phenotypic analysis on tissue sections, St. Marie prepared tissues (Chapter 2.2.2) from the same rabbits, infected with AHV-1, OHV-2 and HipHV-1, used in the experiment described in chapter 3.2 were analysed with immunoperoxidase (Chapter 2.3.1). Serial sections of lymph nodes, spleen, liver, kidney, oesophagus, trachea and lachrymal gland were labelled with anti-CD43 and anti-PCNA (Table 4.1). Cells were considered PCNA positive if labelling was localised in the nucleus. PCNA was also detected in the cytoplasm, which is probably due to the degradation of the protein after cell division. Numeric evaluation was not attempted, because of the high proportion of positive cells and clear pattern of labelling.

4.2.3 FACScan

The spleen and the popliteal lymph nodes of **12 MCF-affected** (Chapter 2.1.3 Pathogenic inoculum: AHV-1) and **6 control animals** (Chapter 2.1.3 Hyperimmunised animals) were examined for the presence of $CD43^+$, $CD5^+$, $CD4^+$, $CD8^+$ and B-cell. The rabbits were killed on the second day of febrile response, and single cell suspensions were prepared from spleen and popliteal lymph nodes (Chapter 2.1.1 and 2.4.6). and FACS analysis was performed (Chapter 2.3.2).

Analysis was performed with a Becton Dickinson FACScan with linear amplification for FSC set at 3.13 V and SSC set at 5.96 V and logarithmic amplification for FITC green fluorescence (FL1) set at 580 PMT Volts. Lymphocytes were distinguished on the basis of the FSC/SSC profile. Two gates (G1 and G2) were set on the basis of a contour profile. The settings and gates were constant for all samples. The phenotype and relative cell size were taken into account, using the formulae:

$$\text{percentage of small cells} = \frac{G1 \times 100}{G1 + G2}$$

$$\text{percentage of large cells} = \frac{G2 \times 100}{G1 + G2}$$

where $G1$ is the number of positive events of small lymphoid cells and $G2$ is the number of positive events of large lymphoid cells. The proportion of large and small leukocytes was calculated for every animal and the mean values of the group compared with each other. Statistical analysis was performed with the Student's t-test.

4.3 Results

All MCF infected animals became febrile after a mean incubation period of 13.6 days (sem 1.04) and showed marked hyperplasia, oedema, necrosis and haemorrhages of the respective lymph nodes (Chapter 3) and splenomegaly. The patterns of lymph node lesions (popliteal and submandibular lymph nodes) were similar in the 12 rabbits used in the FACS analysis. The antigen-stimulated animals only showed slight hyperplasia of the popliteal lymph nodes and granulomatous changes at the site of the inoculation with IFA.

4.3.1 Immunohistochemical analysis of tissues derived from MCF affected and control animals

No difference in labelling patterns was detected between rabbits infected with AHV-1, European OHV-2, Indonesian OHV-2 and HipHV-1 and therefore all will be described together.

I. *Lymphoid tissues*

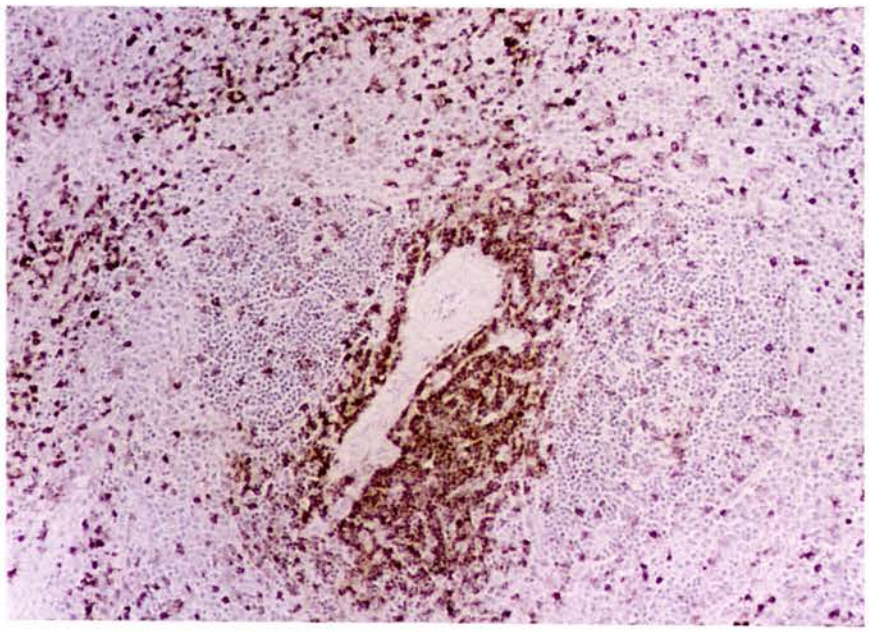
The T-cell areas of spleen (PALS) and lymph nodes (paracortex) were identified morphologically and confirmed by labelling with anti-CD43 in control rabbits (Figure 4.1A and 4.3A). In MCF-affected rabbits, the labelling with anti-CD43 confirmed the hyperplasia of T-cell areas which has been suggested by histological examination (Figure 4.2A and 4.4A). The labelling pattern of anti-PCNA was similar to the pattern obtained with anti-CD43 in spleen (Figure 4.1B and 4.3B) and the affected lymph nodes (Figure 4.2B and 4.4B). Occasionally intrafollicular staining of anti-PCNA could be seen in the spleen. In all control rabbits, only a few single cells were labelled with anti-PCNA.

II. *Non lymphoid tissues*

The histologically observed subepithelial mononuclear lymphocytes present in liver (Figure 4.6), kidney (Figure 4.8), oesophagus, trachea and lachrymal gland of MCF-affected rabbits were almost all CD43 positive. Lymphoid accumulations observed in the lung were also CD43 positive (Figure 4.7). The pattern obtained by labelling with anti-PCNA resembled the pattern obtained with CD43. In liver (Figure 4.5), trachea and lachrymal gland of control rabbits, only few $CD43^+$ cells were found close to the respective epithelia, whereas in the kidney only a few intravascular cells of this phenotype were detected. The $CD43^+$cells found in control rabbits did not stain significantly with anti-PCNA.

Figure 4. 1 Serial sections of a spleen derived from a control rabbit labelled with anti-CD43 (mAb L11/135) and anti-PCNA. The T-cell area is of normal size and only single cells are multiplying.

A- Distribution of $CD43^+$cells around the central arteriole



B- Distribution of $PCNA^+$ cells

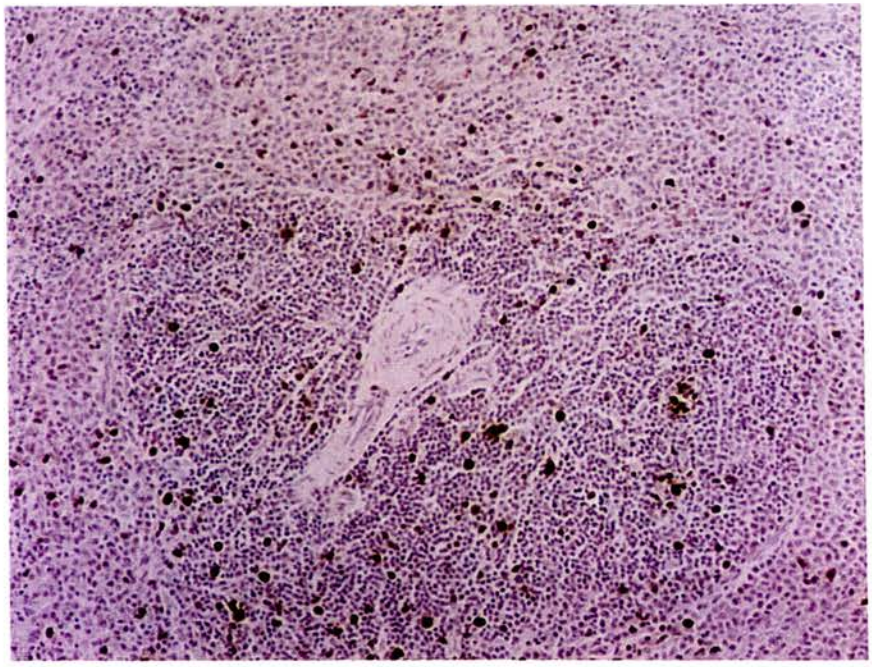
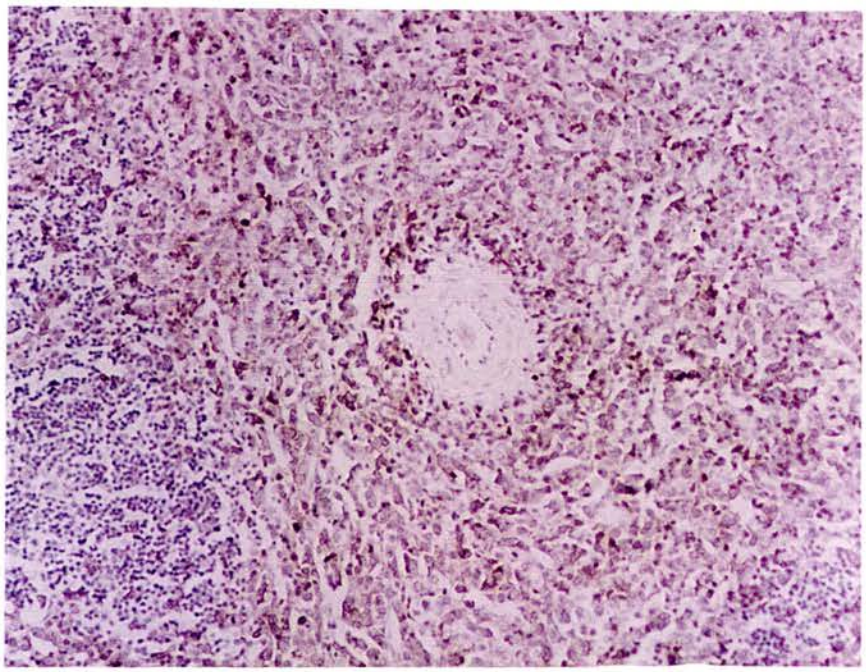


Figure 4. 2 Serial sections of a spleen derived from an OHV-2 infected animal killed at the second day of febrile response labelled with anti-CD43 (mAb L11/135) and anti-PCNA. The T-cell area is expanded and almost all cells are multiplying

A- Distribution of $CD43^{+}$cells around the central arteriole



B- Distribution of $PCNA^{+}$ cells

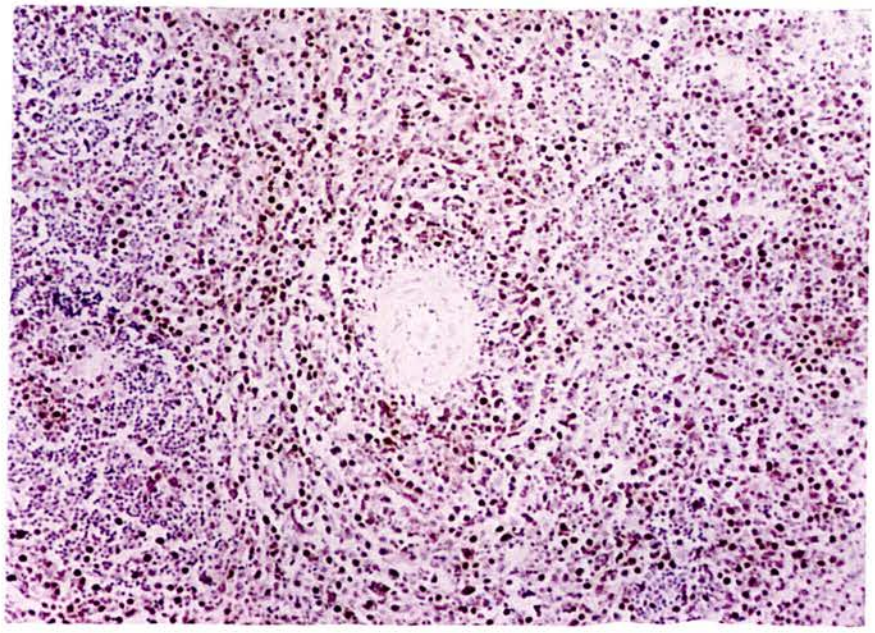
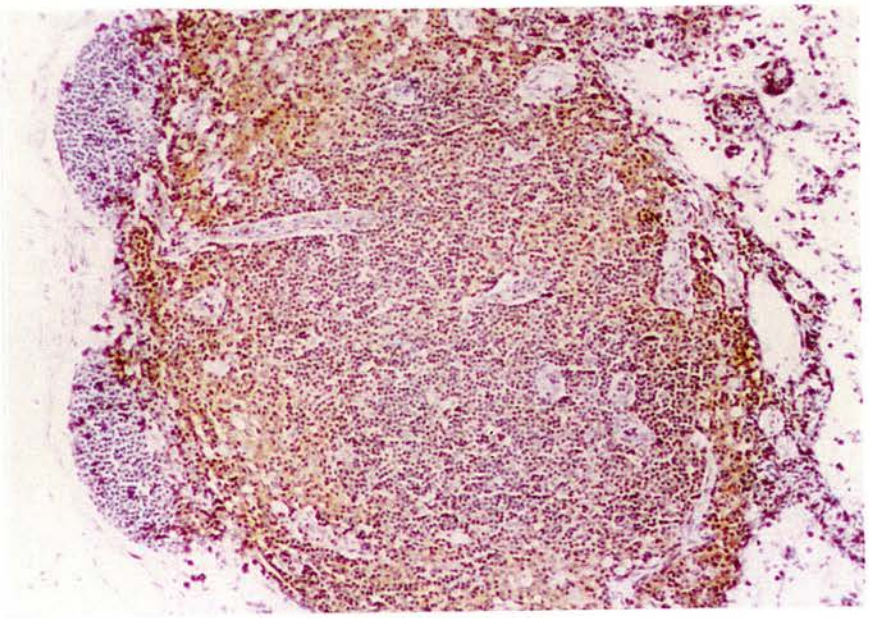


Figure 4. 3 Serial sections of a popliteal lymph node derived from a control rabbit labelled with anti-CD43 (mAb L11/135) and anti-PCNA. The T-cell area is of normal size and only single cells are multiplying.

A- Distribution of $CD43^{+}$cells



B- Distribution of $PCNA^{+}$ cells

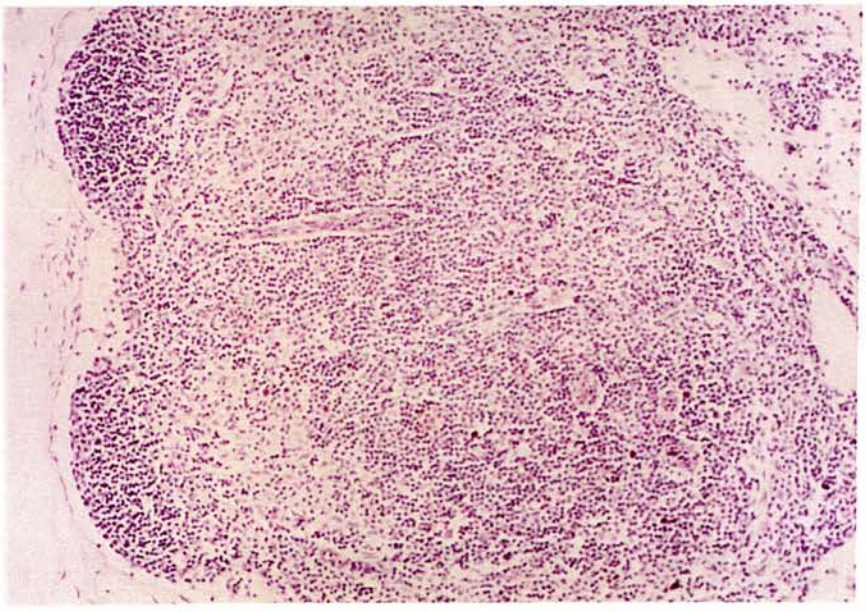
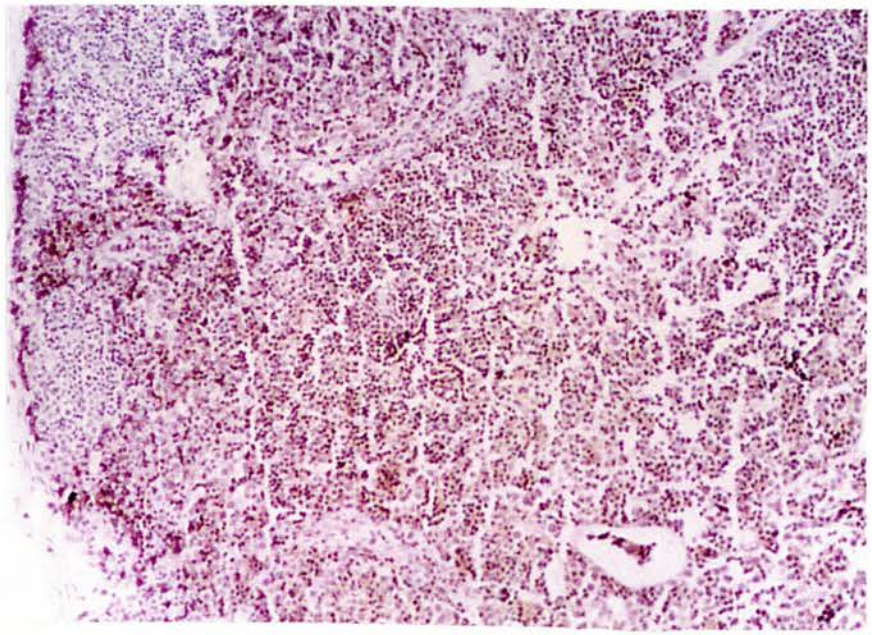


Figure 4. 4 Sections of a popliteal lymph node derived from an AHV-1 infected rabbit killed at the second day of febrile response labelled with anti-CD43 (mAb L11/135) and anti-PCNA. The paracortex is expanded and almost all cells are multiplying.

A- Distribution of $CD43^+$ cells



B- Distribution of $PCNA^+$ cells

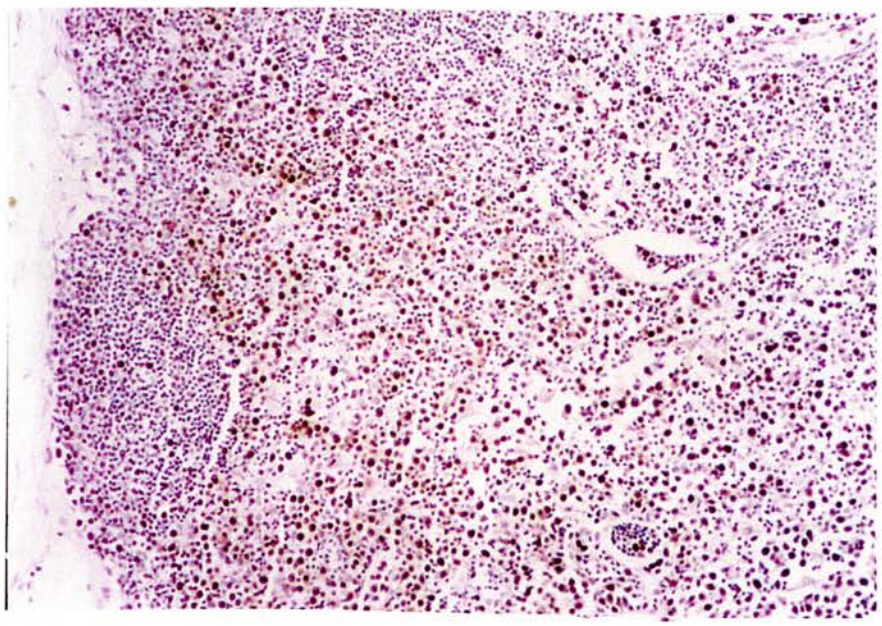


Figure 4. 5 Liver derived from a control rabbit labelled with anti-CD43. Note the single positive cells present in the epithelial layer of the biliary duct

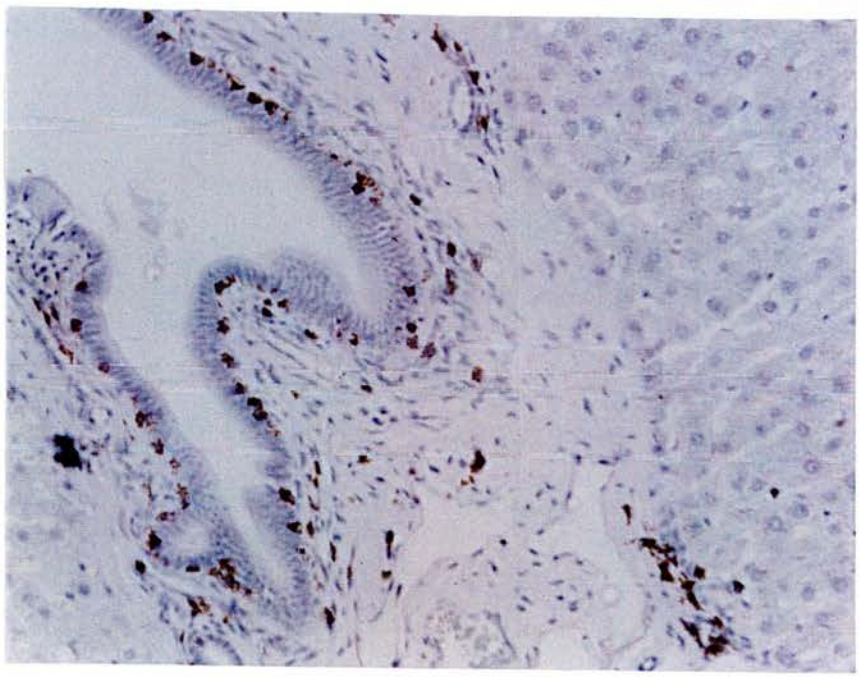


Figure 4. 6 Liver derived from a AHV-1 infected rabbit killed at the second day of febrile response labelled with anti-CD43. Note the increase in number of $CD43^{+}$ cells around the biliary duct.

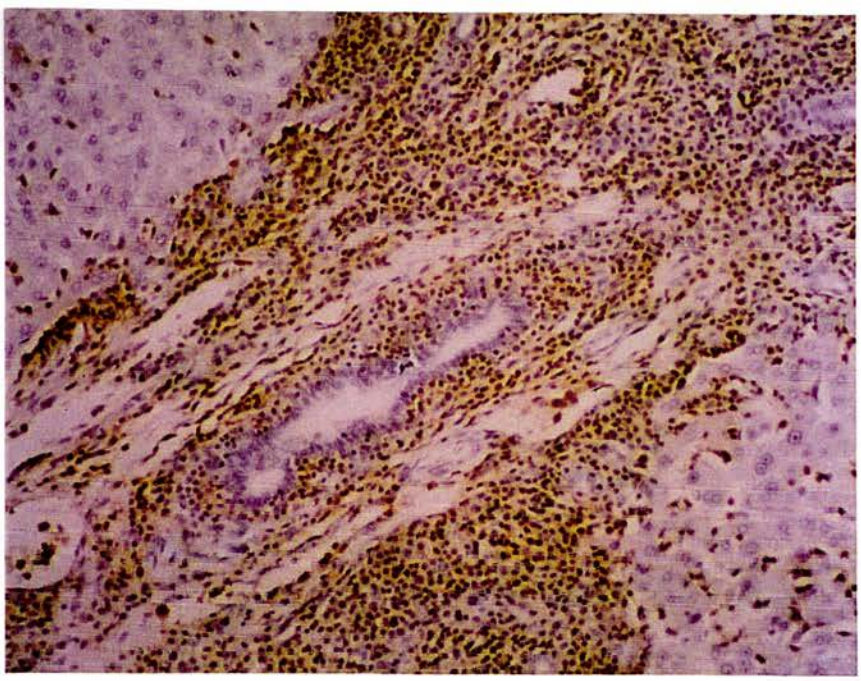


Figure 4. 7 Lung derived from an HipHV-1 infected rabbit on the second day of febrile response stained with anti-CD43.

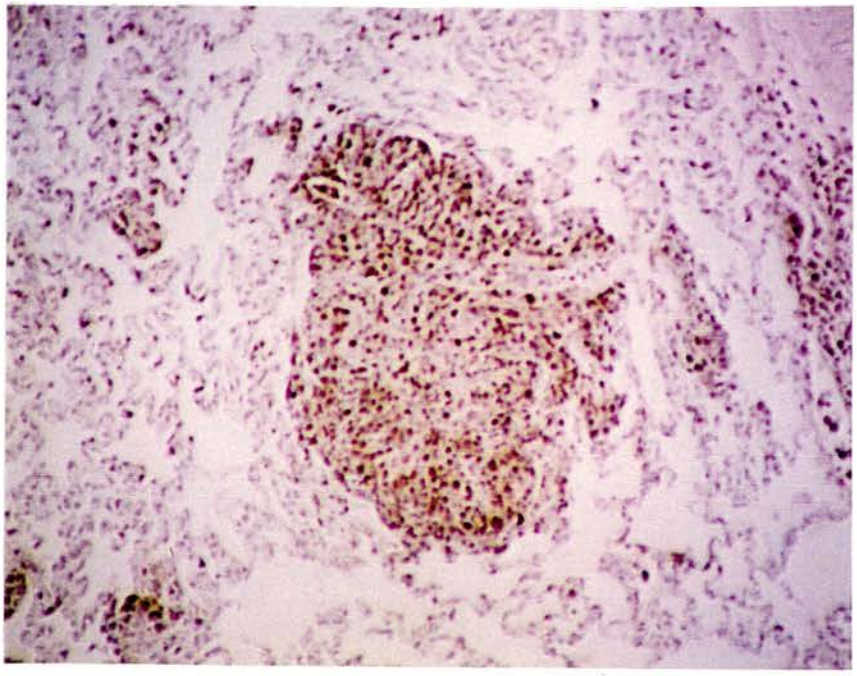
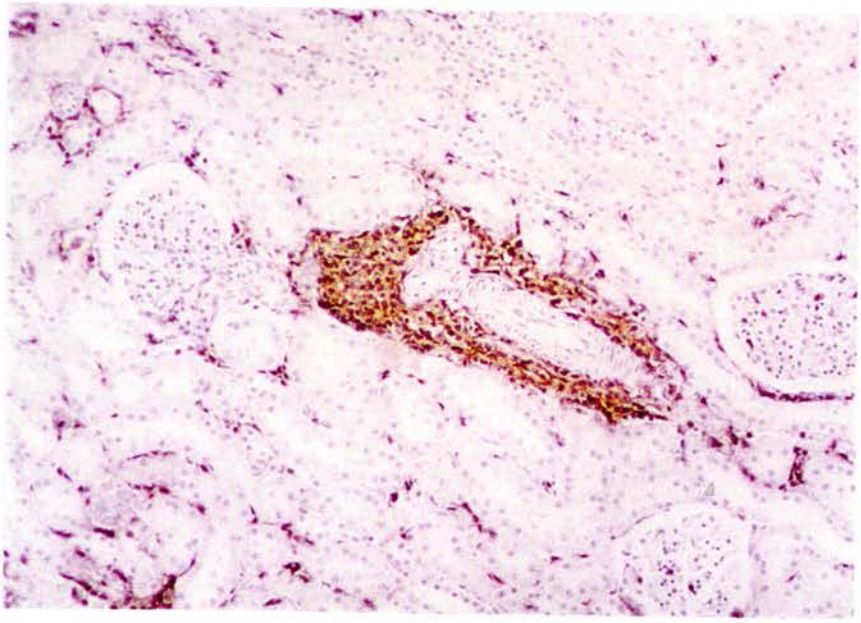


Figure 4. 8 Lymphocyte cell accumulation in the kidney of an AHV-1 infected rabbit killed at the second day of febrile response labelled with CD43.



4.3.2 Comparison of the phenotypes of leukocytes derived from AHV-1 infected rabbits and control rabbits by FACS analysis

The phenotypic analysis of lymphoid cells in spleen (Table 4.2) and popliteal lymph nodes (Table 4.2) showed that $CD43^+$ and $CD5^+$ cells were present in the same proportions in AHV-1-affected animals during the acute phase of the disease as in control rabbits. In the spleen, the proportion of $CD4^+$ cells was comparable in the two groups and no significant difference was observed in $CD8^+$cells (Table 4.2). The proportion of the different subsets detected in control rabbits was comparable to the data published by Okita *et al.* (1995) for control rabbits (Table 4.2).

Comparing cells from popliteal lymph nodes, a significantly greater proportion ($p< 0.05$) of $CD4^+$ cells was detected in control rabbits than in MCF affected rabbits (Table 4.2). Although the tendency towards an increased proportion of $CD8^+$cells in popliteal lymph nodes of MCF affected rabbits was detected, this was not statistically significant. The large variances around the mean value could be the result of the varying extent of hyperplasia found in the experimental groups. Since no author had described the phenotypic analysis of popliteal lymph nodes from rabbits, no reference data were available.

Investigation of the size of the lymphocytes involved in spleen and popliteal lymph nodes indicated that in MCF affected rabbits, $CD5^+$ and $CD8^+$ cells are mainly large, possibly blasting cells ($p < 0.05$) (Figure 4.9a and 4.9D). No such difference between MCF affected and control rabbits was detected for $CD4^+$ and B-cells (Figure 4.9B and 4.9c). A phenotypic comparison of splenocytes from a control rabbit examined immediately *ex vivo* and Con A blasts showed that the size of lymphocytes increased upon stimulation, which supports the hypothesis that the size of lymphocytes reflects their blastogenic activity.

Table 4. 2 Comparison of the phenotypes of leukocytes derived from AHV-1 infected and control rabbits

A- Spleen

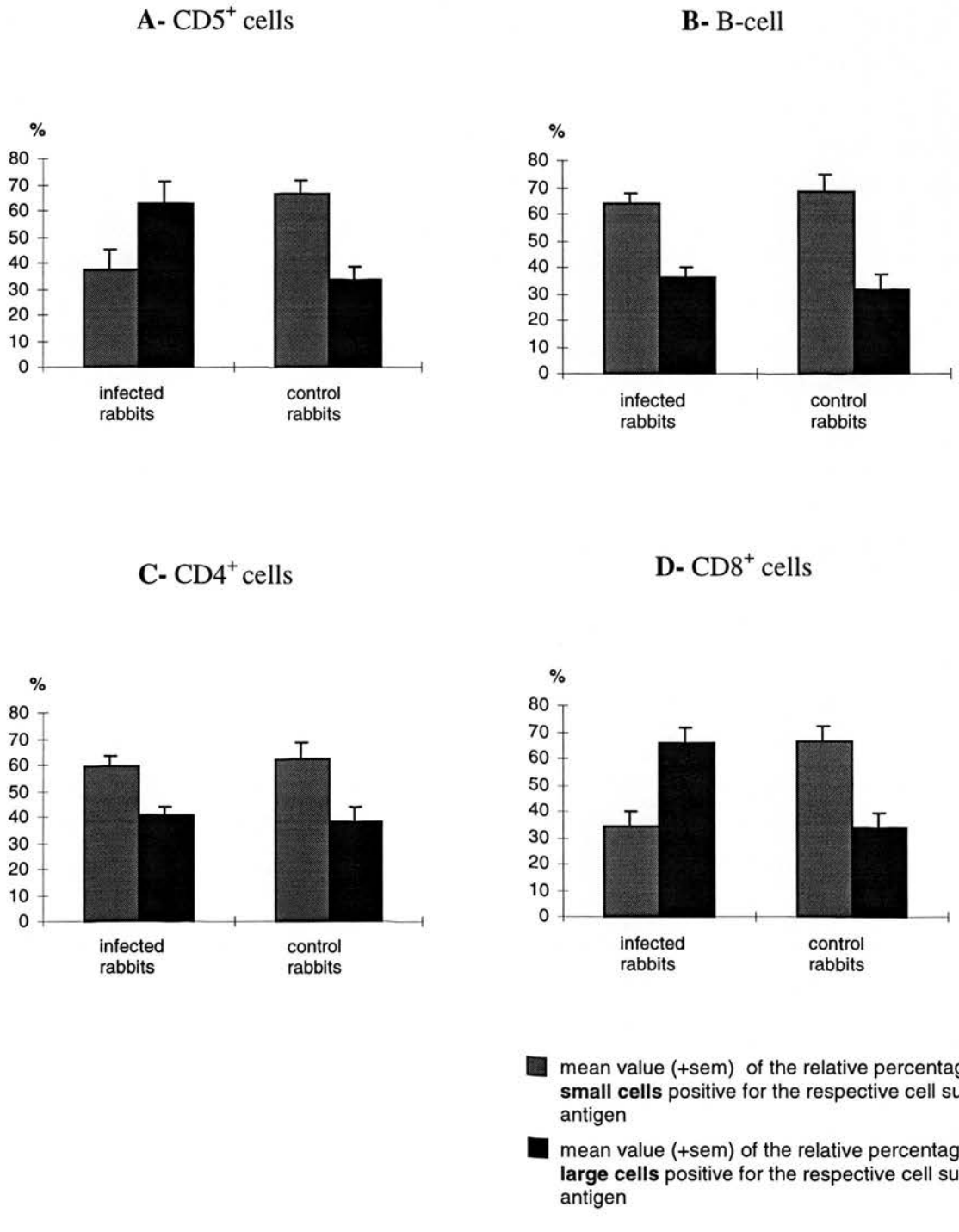
mAb	MCF-infected n = 12	Control n = 6	reference[2]
CD43	55.5[1] ± 5.5	55.4 ± 3.0	nd
CD 5	56.7 ± 7.6	48.4± 3.5	46.9
CD 4	21.0 ± 3.2	22.3 ± 3.8	28.5
CD 8	34.4 ± 5. 5	23.6 ± 7.0	nd
NRBM	41.0 ± 6.4	32.7 ± 2.8	nd
anti-IgG	34.7 ± 3.1	31.6 ± 2.8	29.0

B- Popliteal Lymph Node

mAb	MCF-infected n = 12	Control n = 6
CD43	60.5[1] ± 4.2 [2]	62.6 ± 4.3
CD 5	58.5 ± 3.4	58.3 ± 3.5
CD 4*	28.7 ± 2.9	48.4 ± 2.2
CD 8	31.5 ± 4.2	17.7 ± 6.9
B-cell	36.2 ± 5.2	20.5 ± 4.9
anti- IgG	31.7 ± 4.6	26.4 ± 3.6

[1] mean value ± sem of the percentage of positive cells [2] proportion found by Okita *et al.* (1995) in six control rabbits; **nd** not determined; *$p < 0.05$ (student's T-test)

Figure 4. 9 Comparison of the relative size (Chapter 4.2.3) of leukocytes derived from the popliteal lymph nodes of rabbits infected with pathogen AHV-1 and control rabbits



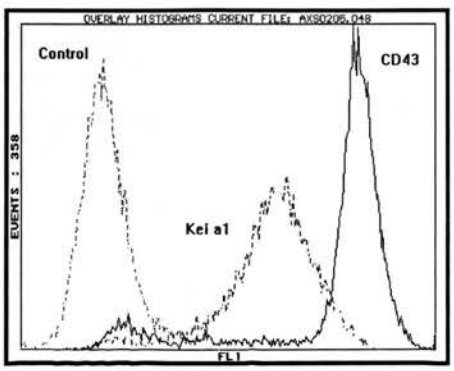
4.3.3 Kei α1 as a marker for IL-2R expression

The available marker for cell activation, CD25 (IL-2Rα) gave good results with the IL-2 dependent cell line, but did not give satisfactory results with cells from the MCF affected animals (Figure 4.10). Only a few cells from affected and control animals were positive for this mAb, if stained directly *ex vivo*. This finding did not seem genuine, because lymphocytes prepared in the same way clearly respond immediately to exogenous IL-2 in culture and should therefore possess functional IL-2R (Chapter 5).

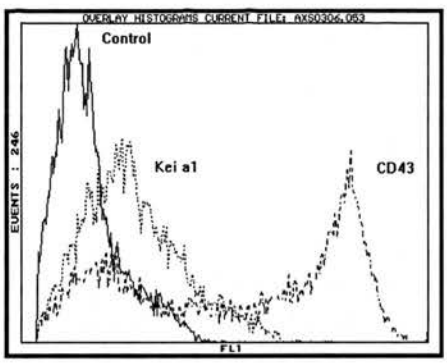
Only a few splenocytes derived from the antigen stimulated rabbits were positive for Kei α1 when tested directly after the post mortem. After 48 hours stimulation with Con A, the proportion of large cells increased (Figure 4.11) and 80% were positive with almost no change in the proportions of $CD43^+$ cells (Figure 4.10). No alternative mAb was available.

Figure 4. 10 Evaluation of the mAb Kei α1 (anti-rabbit CD25) (dilution 1:1000) and CD43 in FACScan on rabbit leukocytes

A- Cell line BJ 1008 after 48 hours stimulated with hrIL-2



B- Splenocytes derived from an hyperimmunised rabbit



C- Splenocytes derived from the same rabbit stimulated for 48 hours with 5μg/ml Con A

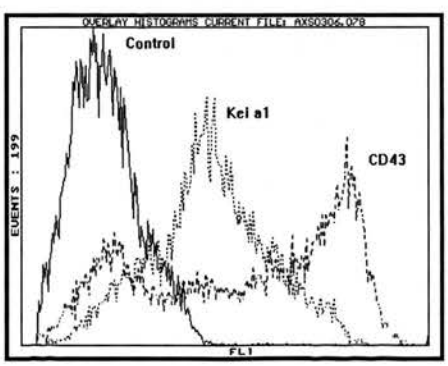
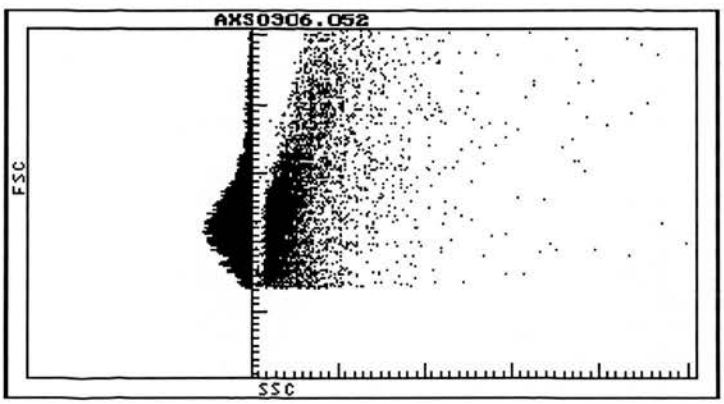
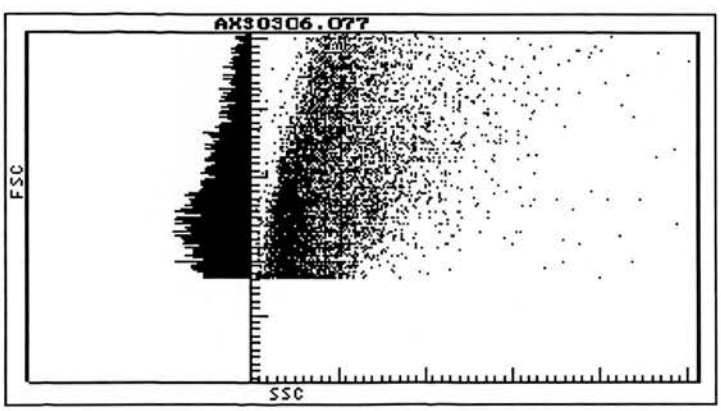


Figure 4. 11 Comparison of the size of unstimulated and Con A stimulated lymphocytes derived from a healthy rabbit

A- Splenocytes derived from an healthy rabbits



B- Splenocytes derived from the same rabbit after 48 hours in culture with 5 μg/ml Con A



4.4 Discussion

4.4.1 Mononuclear lymphocyte accumulations: actively multiplying T-cells?

The hypothesis that the mononuclear lymphocyte accumulations observed in mucosal sites and kidney in rabbits experimentally affected with MCF are due to an accumulation of T-cells was confirmed by labelling the cells with a T-cell specific mAb. This confirms the finding by Buxton *et al.* (1984) who used a polyclonal anti-rabbit T-cell serum to characterise the lymphoid cells involved in the pathogenesis of OHV-2 infection in rabbits.

PCNA is expressed in cells during the S-phase of their multiplicative cycle (4.2.1) and it can therefore be assumed that $PCNA^+$ cells are actively multiplying. It was shown in serial sections that the T-cell accumulations observed in experimental MCF are PCNA positive. The labelling with anti-PCNA mAb and the increase in numbers of T-cells at mucosal sites in animals affected with MCF strongly suggest, that the T-cells are actively multiplying *in situ*. The presence of single $CD43^+$ cells in liver (also shown by Wilkinson *et al.* 1993), trachea and lachrymal gland in control rabbits suggests that a resident T-cell population exists in non-lymphoid tissues, which might be stimulated to multiply during the course of MCF. It is probable, therefore, that only a minority of the cells accumulating in these tissues derive from infiltration, whereas in the kidney and the lung, they are likely to be recruited from the circulation. Since there is little evidence for viral expression in these kind of lesions it must be hypothesised, that this T-cell multiplication is either due to the chemotactic effect of resident APC or to the release of a systemic T-cell activator. This does not support the suggestion made by Reid and Buxton (1989), that the distinct distribution patterns of MCF lesions induced by the different viruses was due to infection of a specific lymphocyte subset with different homing characteristics, since *in situ* T-cell multiplication was present in all four groups in the various organs, with the exception of kidney and lachrymal gland.

4.4.2 Hyperplasia of spleen and lymph nodes

Histological and immunohistochemical examination of spleen and lymph nodes showed that most of the hyperplasia was due to the expansion of T-cell dependent areas. In contrast, the phenotypic analysis of spleen and lymph node cells derived from AHV-1 infected rabbits, showed that the **overall proportion** of the different subsets was maintained. Surprisingly, there was no alteration in the proportions of T- and B- cells between MCF affected and hyperimmunised rabbits as had been expected. However, a decrease of $CD4^+$ cells in the popliteal lymph nodes and a relative increase of large $CD5^+$ and $CD8^+$ cells in spleen and lymph nodes was detected. Furthermore, rabbits were killed on the second day of the febrile response (acute phase of the disease) and therefore three different phenomena overlap:

1. the pathological hyperplasia induced by the virus
2. a physiological immune response to foreign antigens (viral antigen and/or antigens present in the inoculum)

and 3. necrosis

It is not possible to segregate the different events, since the FACS analysis only shows the phenotype of viable cells, not the mechanism involved in the pathogenesis. The results have therefore to be interpreted with caution. The data obtained could reflect the expression of pathological events, but technical problems may also play a role.

Apoptosis and cytotoxicity The FACScan was performed on lymph node cells which were prepared by gradient centrifugation. This technique only enumerates viable cells and does not register dying cells which are detected on histological examination. Furthermore, short term cultures of similarly prepared cells derived from lymph node and spleen of rabbits experimentally infected with AHV-1 showed a large proportion of dying cells over the first 72 hours in culture (Chapter 5.3.2). These observations lead to the hypothesis, that the FACScan does not reflect the observed hyperplasia because the dead cells which may either represent all phenotypes or, as observed histologically, only T-cells. Retrospective appraisal suggests that it would have been better to have analysed tissues taken from animals

during the incubation period of the disease. It would, however, have been difficult to establish the optimum time point after infection at which to do the FACScan and the resulting numbers of animals required would have been much greater. Furthermore, large variance would have been expected. Because of the limited amounts of mAbs available, this experiment was not undertaken.

It has yet to be established if the dead cells observed in the lesions undergo apoptosis or die as a consequence of cytotoxic cells. The latter hypothesis was suggested by the observation that cell lines derived from MCF affected animals are cytotoxic (Cook and Splitter, 1988; Reid *et al.* 1989). Therefore, it would have been useful to have included markers for programmed cell death such as Bcl-2 and Bcl-X in the panel of mAbs, which were not available, to investigate whether or not spleen and lymph node derived lymphocytes undergo apoptosis (reviewed by Nunez *et al.* 1994).

T-cell activation in spleen and lymph node during MCF The histological data suggests that the relatively low proportion of $CD4^+$ cells in the popliteal lymph nodes of MCF-affected rabbits, the increased size of the $CD5^+$ and $CD8^+$cells, and the large variance in the proportions of $CD8^+$cells could indicate, that the hyperplasia observed during MCF is mainly due to $CD8^+$ cells. The increase in size observed in Con A blasts strengthens the hypothesis that size is connected with the transformation into blasting cells.

These data would confirm data of other authors that the lymphoid hyperplasia in MCF affected animals is mainly due to $CD8^+$ T-cells (Ellis *et al.* 1992; Nakajima *et al.* 1992).

B-cell stimulation during MCF The comparable proportion of B-cells in MCF affected and hyperimmunised rabbits can be explained as the physiological reaction to foreign antigen such as viral antigens, splenocytes and components of the cell culture medium present in the inoculum. For example, WA-MCF affected rabbits produce antibodies to AHV-1 which can be detected by IF/IIF four to five days before the onset of pyrexia (Rossiter *et al.*, 1977, 1978, 1982). In addition, it can be hypothesised that cell types other than T-cells are also implicated in the acute phase of the disease.

CD25 expression of lymphocytes in MCF affected animals The staining with anti-CD25 did not give convincing results and it could not be established if cell populations from MCF affected rabbits had a higher expression of IL-2Rα (Chapter 4.3.3). As mentioned before, freshly explanted lymphocytes derived from lymph nodes of MCF affected rabbits react readily to exogenous rIL-2 and it can therefore be assumed that these cells bear a functional IL-2R. The lack of staining with Kei-1α could be due to the inadequate characterisation of the mAb which was developed against HTLV-1 immortalised cell lines (Kotani *et al.* 1993). Since the antibody gave satisfactory results on IL-2 dependent AHV-1 immortalised cell lines, precipitates a protein of circa 55 kDa and was shown to have neutralising activity *in vitro* (Kotani *et al.* 1990), it can be assumed that the mAb recognises the rabbit equivalent of the murine and human IL-2Rα.

It is possible that staining of synchronised cell lines is different from staining of freshly explanted leukocytes in which the IL-2Rα may be occupied or internalised. A further possibility could be that while the other subunits of the IL-2R are expressed, IL-2Rα is not present at a high frequency (Chapter 1.3.2). Since the other subunits are not inducible and are constitutively expressed it is not likely that their expression is significantly altered.

Overall, the results obtained by immunocytochemistry strengthen the hypothesis of various authors, that the principal cells involved in the pathogenesis of MCF are T-cells (reviewed by Reid and Buxton, 1989), even though other cell types may be involved in the acute phase of MCF. The apparent *in situ* multiplication of T-cells in mucosal sites, the increase in size of $CD4^+$ and $CD8^+$ cells in lymphoid organs together with the lack of detectable viral DNA suggest, that a small lymphocyte subset, or perhaps antigen presenting cells such as macrophages and dendritic cells, are infected by the given MCF virus. The homeostasis of the lymphoid system may be altered by dysregulating a lymphocyte activator such as interleukin-2, which is either acting sytemically inducing the cell multiplication in lymphoid and non lymphoid tissues or is dysregulated locally in every affected site (Chapter 1.3).

4.5 Summary

1. The lymphoid cell accumulations in non lymphoid tissue, characteristic for MCF, are mainly *in situ* multiplying T-cells.
2. The proportions of lymphocyte subsets in spleen and popliteal lymph nodes were maintained in WA-MCF affected rabbits with the exception of $CD4^+$ cells which were reduced in the popliteal lymph nodes compared to hyperimmunised rabbits
3. $CD5^+$ and $CD8^+$ cells were enlarged in spleen and popliteal lymph nodes of WA-MCF affected rabbits compared to hyperimmunised animals.

Chapter 5

Short term cultures of lymphocytes derived from MCF-affected rabbits

5.1 Introduction

The pathological and immunocytochemical analysis of peripheral lymph nodes in AHV-1 infected rabbits, demonstrated extensive T-cell hyperplasia in these organs (Chapter 3 and 4). These data together with the lack of viral expression, led to the conclusion that lymphocytes are severely dysregulated during the course of MCF (Chapter 1.2.6). In addition, Mushi and Rurangirwa (1981, c) showed that specific delayed cutaneous hypersensitivity to AHV-1 antigens was not detectable during clinical disease in rabbits, even though it could be induced during the incubation period. These data indicate that lymphocytes may be reacting physiologically during the incubation period of MCF and that the dysfunction of the immune system occurs during the febrile phase of the disease. To investigate the dynamics of this dysregulation, the growth of lymph node cells derived from febrile MCF-affected rabbits was studied.

Freshly explanted lymphocytes were cultured with medium alone, medium containing Concanavalin A (Con A) or medium containing human recombinant IL-2 (hrIL-2) and their growth assessed by conventional cell counts and by incorporation of tritiated thymidine.

It was hypothesised, that the growth of lymph node cells in medium would reflect the hyperplasia occurring *in vivo*. The T-cell mitogen Con A (Anderson *et al.,* 1971; Janossy and Greaves, 1971) was primarily included as a positive control. The lymphocytes were stimulated with exogenous IL-2 to determine whether or not, in the absence of IL-2, these cells would be able to produce enough IL-2 to sustain their own growth. This was of particular importance with regard to the hypothesis that the MCF-associated hyperplasia is caused by a virus-induced hyperproduction of IL-2.

Lymphocytes derived from rabbits with MCF following infection with AHV-1 were compared with cells derived from animals infected with non pathogenic AHV-1 and animals which had not been exposed to virus, cells or medium. It was expected that the comparison between these three groups and the various culture conditions would elucidate differences between the behaviour of lymphocytes derived from MCF-

affected animals and lymphocytes involved in the self-limiting physiological hyperplasia to foreign antigen.

5.2 Materials and methods

5.2.1 Preliminary experiment

Rabbits infected with an inoculum containing pathogenic AHV-1 were monitored clinically, and killed on the second day of febrile response (Chapter 2.1). Single cell suspensions were prepared from spleen, popliteal and submandibular lymph nodes (Chapter 2.4.6). Initial experiments established the optimal cell concentration for cell growth in 24-well cell culture plates as being $2x10^6$ cells/ml. Lymphocytes derived from four animals were cultured at $2x10^6$ cells/ml with CM alone and with 300 U/ml hrIL-2 in a volume of 2 ml. The cells were harvested, diluted in trypan blue and counted with a haemocytometer at 0, 24, 48, 72 and 144 hours. The total number of cells was recorded as the sum of live (transparent) and dead (stained) cells. The viability (**V**) is expressed as the percentage of live cells:

$$V = \frac{LC}{LC + DC} \times 100$$

where **LC** is the number of live cells and **DC** is the number of dead cells

5.2.2 Cell growth of lymphocytes derived from AHV-1 infected animals evaluated by incorporation of thymidine

Seventeen rabbits were allocated to three groups. Group 1 of eight animals was inoculated with rabbit spleen cells infected with **pathogenic** AHV-1 and killed on the second day of febrile response (Chapter 2.1). Group 2 of four animals was inoculated with cells infected with **apathogenic** derivatives of AHV-1 (Chapter 2.1) and one animal was injected with cell culture medium alone in a total volume of 1 ml (Chapter 2.4.2). Group 3 consisted of four animals which were not inoculated. All control animals were monitored clinically and killed 12 weeks after beginning of the experiment.

Both submandibular and both popliteal lymph nodes were removed from each rabbit and single cell suspensions prepared (Chapter 2.4.6). The cell concentration was established using an haematocytometer. Cells were added at 2 x 10^5 cells/well to

each of three 96-well-plates to be harvested at 24, 48 and 72 hours. On each plate the cells were cultured with CM alone (Chapter 2.4.2), or in CM supplemented with 150 U/ml rhIL-2 (Chapter 2.4.5) or 5 µg/ml Con A (Chapter 2.4.5) in a volume of 200 µl. Lymphocytes derived from animals of group 1 were incubated with 300, 150, 75, 37.5 and 18.75 U/ml rhIL-2. Splenocytes from one animal (AS 93/27) were incubated with the same concentrations of recombinant bovine IL-2 (brIL-2) to further compare responses to a non-homologous cytokine. The thymidine uptake was determined as described in (Chapter 2.4.7). The counts per minute (cpm) were calculated for cultures of each animal and the final data is expressed as the group mean cpm.

The increase of the thymidine uptake due to exogenous IL-2 was expressed by the mean stimulation index for IL-2 (SI_{IL2}) which was calculated as the mean value of the stimulation index for every animal:

$$SI_{IL2} = \frac{cpm(IL2)_1/cpm(med)_1 + \ldots + cpm(IL2)_n/cpm(med)_n}{n}$$

where cpm (med) is the cpm of culture with medium alone, cpm (IL2) is the cpm of IL-2 supplemented cultures and n is the number of animals in one group.

The statistical significance was calculated with four-way analysis of variance (ANOVA). In contrast to the simple Student's t-test, this statistical method takes in consideration when a variety of measurements had been made on more then two groups taking into account the interaction of the different measurements (Bishop, 1971).

5.3 Results

5.3.1 Pathology

All rabbits injected with pathogenic AHV-1 (group 1 and rabbits used for the preliminary experiment) became febrile in a mean incubation period of 9.37 days (sem = 0.18). Popliteal and submandibular lymph nodes were markedly hyperplastic and haemorrhages were common. Spleens were very enlarged, dark in colour and with prominent white pulp. There were small white spots on the surface of the kidneys in two infected rabbits. These lesions are consistent with the diagnosis of MCF. Animals of group 2 did not develop fever for at least 12 weeks and did not show any macroscopic lesion, nor did animals of group 3.

5.3.2 Preliminary experiment

The cell counts of freshly explanted lymphocytes from four AHV-1 affected rabbits over 144 hours showed that the total number of cells significantly increased ($p < 0.001$) and that IL-2 enhanced ($p < 0.001$) the growth of these cells (5.1 A). In contrast, the source of the cells did not have any significant effect. The percentage of live cells in cultures with medium alone and with hrIL-2 decreased ($p < 0.001$). Nevertheless, the viability was enhanced by hrIL-2 ($p < 0.01$) (Table 5.1 B).

Table 5. 1 Short term cultures of leukocytes derived from spleen, popliteal and submandibular lymph nodes of AHV-1 infected rabbits cultured with medium alone and supplemented with rhIL-2 (300U/ml)

A- Total number of cells x 10^6/ml

Organ	CC	0 hours	24 hours	48 hours	72 hours	144 hours
spleen	CM	2.3[1] ± 0.3	2.7 ± 0.5	2.8 ± 0.5	4.3 ± 0.8	4.5 ± 1.2
	IL-2	2.3 ± 0.3			4.0 ± 0.8	6.2 ±1.1
pLN	CM	2.4 ± 0.2	3.7 ± 0.3	5.0 ± 0.6	5.2 ± 0.8	5.9 ± 1.0
	IL-2	2.4 ± 0.2			6.3 ± 0.9	10.3 ± 2.0
sLN	CM	2.1 ± 0.1	4.2 ± 0.2	5.1 ± 0.3	7.0 ± 0.6	6.7 ± 0.7
	IL-2	2.1 ± 0.1			6.5 ± 0.8	8.6 ± 1.5

B- Viability

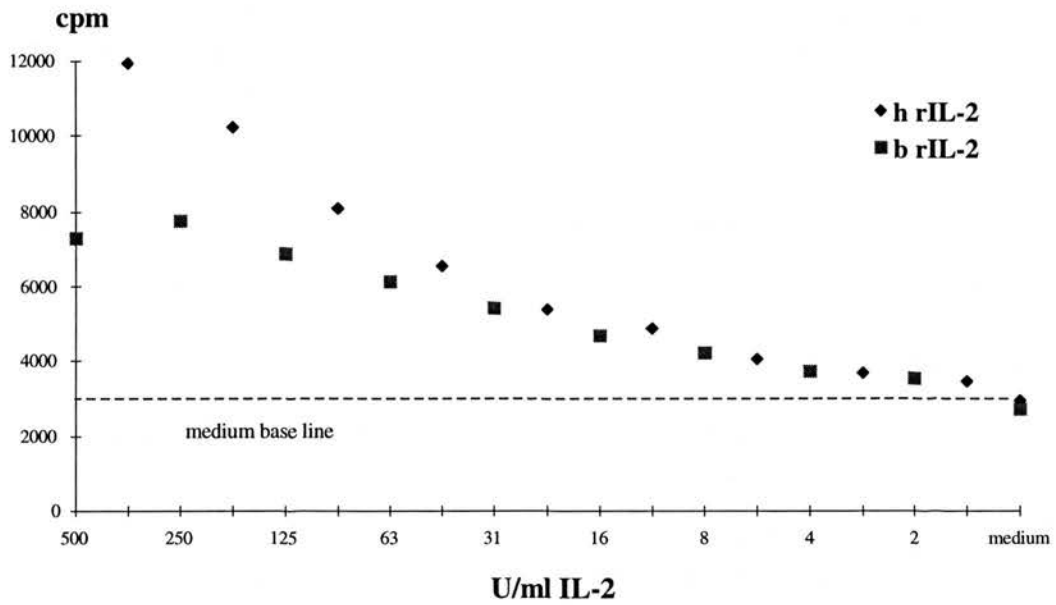
Organ	CC	0 hours	24 hours	48 hours	72 hours	144 hours
spleen	CM	100[3]	38.7 ± 6.4	43.2 ± 4.5	31.7 ± 3.7	17.8 ± 4.5
	IL-2	100			45.3 ± 6.4	31.5 ± 9.3
pLN	CM	100	56.3 ± 6.5	47.6 ±7.8	43.7 ± 3.0	34.5 ± 5.4
	IL-2	100			53.5 ± 3.8	48.1 ± 7.2
sLN	CM	100	53.1 ± 6.6	51.7 ±5.1	40.9 ± 5.4	25.3 ± 10.77
	IL-2	100			49.1 ± 11.3	39.4 ± 14.4

CC: culture conditions; **CM**: culture medium alone; **IL-2**: cultured with hrIL-2; **pLN**: popliteal lymph node; **sLN**: submandibular lymph node; [1] mean value ± sem [2] percentage of live cells (mean value ± sem)

5.3.3 A comparison of the effect of recombinant human and bovine IL-2

The effect of different concentrations of hrIL-2 and brIL-2 in the culture medium on the thymidine uptake after 48 hours incubation is shown in figure 5.1. Differences in thymidine uptake at a given cytokine concentration were not statistically significant.

Figure 5. 1 Comparison of the effect of human and bovine recombinant IL-2 on the thymidine uptake of splenocytes derived from an AHV-1-affected rabbit incubated for 48 hours



5.3.4 Thymidine uptake by lymphocytes derived from AHV-1 infected rabbits evaluated by incorporation of tritiated thymidine

The four-way ANOVA, showed a significant difference ($p < 0.001$) in thymidine uptake between the three groups, the time and the different culture condition whereas no significant difference could be detected between the thymidine uptake of cells derived from popliteal and submandibular lymph nodes. The specific data as well as the single statistical interaction are described below. They are summarised in table 5.2, and represented in figure 5.2.

Cultures with medium alone Popliteal lymph node cells (PLNC) derived from animals of **group 1** had a mean cpm of 11,404 (SEM =1,573) in the first 24 hours. This value fell ($p = 0.01$) to 5,520 cpm (SEM = 1,511) after 3 days of culture. The cpm of animals from **group 2** were low 2,517 cpm (SEM = 408) at 24 hours and increased ($p < 0.01$) to 14,382 cpm (SEM = 4,315) at 72 hours. The thymidine uptake of PLNC of not infected animals (**group 3**) remained low throughout the 72 hours (Table 5.2).

Cultures supplemented with human rIL-2 PLNC cultured with rhIL-2 from **group 1** had a mean cpm of 15,995 (SEM = 1,694) in the first 24 hours. This value decreased ($p < 0.01$) to 10,640 (SEM = 1,053) after 72 hours in culture. The mean cpm of PLNC from **group 2** increased ($p < 0.01$) from 3,075 cpm (SEM = 506) in the first 24 hours to 19,300 cpm (SEM = 4,664) at 72 hours. The mean cpm for PLNC derived from **group 3** was increasing over the three days of culture from 2171 cpm (SEM = 651) for 24 hours to 8,042 cpm (SEM = 2,997) at 72 hours. (Table 5.2 A). The values for the submandibular lymph node cultures were of the same order (Table 5.2 B).

Cultures supplemented with Con A The mean thymidine uptake of PLNC cultures supplemented with Con A **group 1** cultures remained unaltered over the 72 hour period (Table 5.2 A). There was no significant difference between the thymidine uptake of cultures with medium alone and supplemented with Con A in these rabbits. This contrasts with the values for **group 2 and 3** which increased significantly ($p < 0.01$) over 48 hours (ca 30,000 cpm) and declined slightly at 72 hours (Table 5.2 A). The thymidine uptake of cultures with medium supplemented with Con A was higher ($p < 0.001$) than the uptake by cells incubated with medium alone. The values for the submandibular lymph node cultures were very similar and are shown in table 5.2 B.

Table 5. 2 Thymidine uptake by lymphocytes derived from rabbits which were either not inoculated (Group 3) or infected with pathogenic (Group 1) and apathogenic AHV-1 (Group 2) at 24, 48 and 72 hours cultured with medium alone, medium supplemented with 150 U/ml hrIL-2 and 5 μg/ml Con A

A- Popliteal lymph node cells

Group	time	24 hours	48 hours	72 hours
1	medium	11,404[1] ± 1,573 [2]	9,019 ± 2,392	5,520 ± 1,511
	IL-2	15,995 ± 1,694	14,005 ± 1,583	10,640 ± 1,053
	Con A	9,867 ± 1,136	9,881± 2,440	8,763 ± 2,282
2	medium	2,517 ± 408	7,474 ± 1,824	14,382 ± 4,315
	IL-2	3,075 ± 506	10,119 ± 1,920	19,300 ± 4,664
	Con A	13,403 ± 2,364	37,904 ± 2,784	25,986 ± 1,444
3	medium	1,008 ± 226	680 ± 77	811± 143
	IL-2	2,171 ± 651	4,133 ± 1,380	8,042 ± 2,997
	Con A	13,421 ± 3,018	37,069 ± 748	31,289 ± 1,068

B- Submandibular lymph node cells

Group	time	24 hours	48 hours	72 hours
1	medium	10,315[1] ± 989 [2]	9,100 ± 2107	5,402 ± 1245
	IL-2	14,668 ± 965	13,365 ± 1,248	9,878 ± 1,178
	Con A	10,120 ± 1,067	8,656 ± 1,813	6,541 ± 1,745
2	medium	1,767 ± 508	6,374 ± 1,277	8,695 ± 3,800
	IL-2	2,434 ± 545	5,348 ± 1,758	11,214 ± 4,138
	Con A	8,209 ± 2,583	28,475 ± 7,455	24,435 ± 1,956
3	medium	1,057 ± 306	872 ± 339	998 ± 435
	IL-2	2,064 ± 588	3,006 ± 1,531	5,827 ± 4,266
	Con A	12,605 ± 4,643	33,083 ± 2,954	23,852 ± 3,827

[1] mean cpm ± sem;

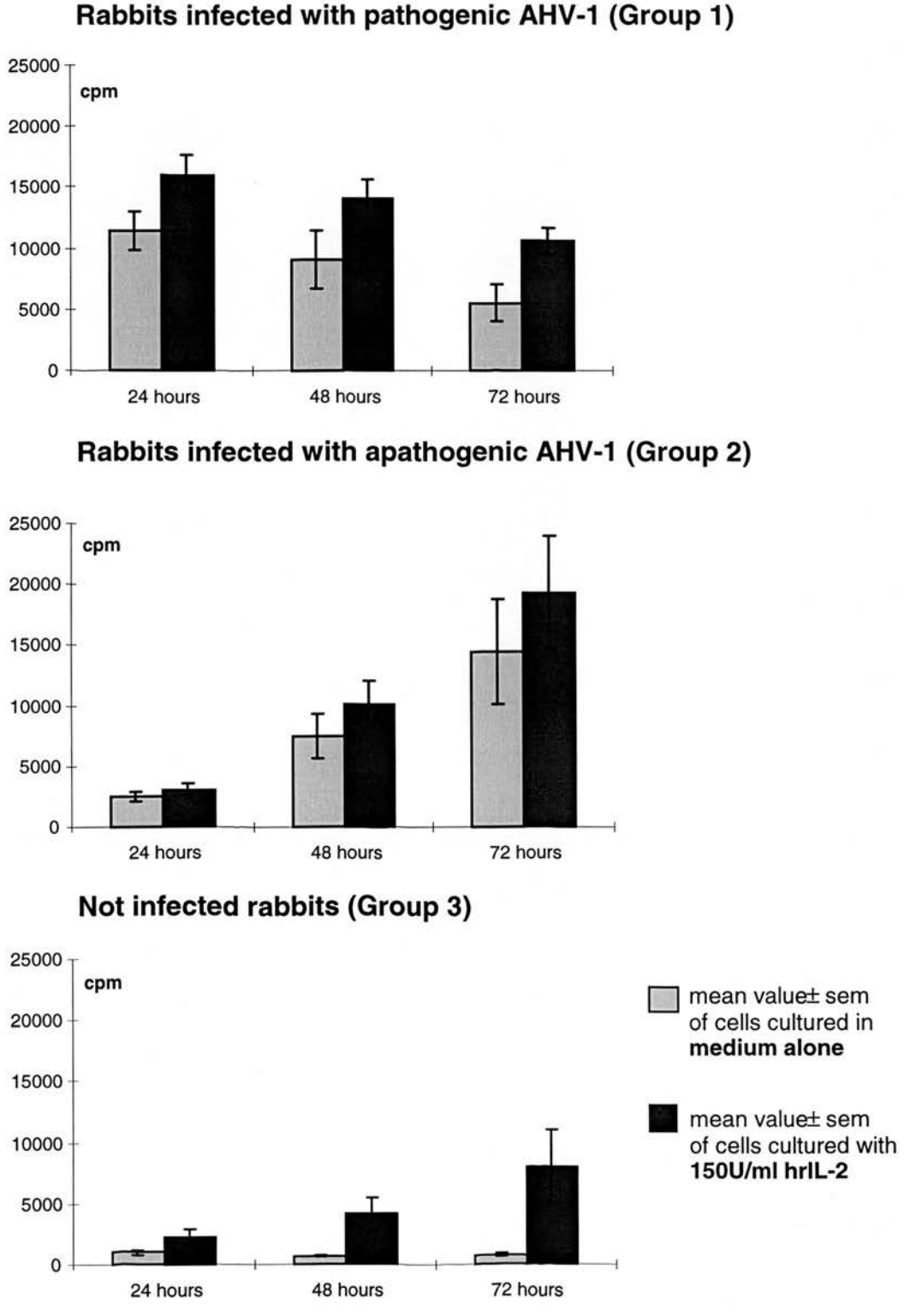
IL-2 stimulation index (SI_{IL2}) The statistical analysis for this parameter was calculated separately by four-way ANOVA. A significant increase ($p < 0.001$) in thymidine uptake was found between medium alone and rhIL-2 supplemented cultures in all groups (Figure 5.2). The ratio varied significantly in popliteal and submandibular lymph nodes ($p < 0.01$) between the groups (Table 5.3). Whereas the ratio in **group 1 and 3** increased significantly ($p \leq 0.01$) over time, the ratio of **group 2** did not increase.

Table 5. 3 IL-2 stimulation index (SI_{IL2}) of lymphocytes derived from popliteal lymph nodes of rabbits experimentally infected with AHV-1

Group	24 hours	48 hours	72 hours
Group 1 (pathogenic inoculum)	1.5 [1]	2.8	4.9
Group 2 (apathogenic inoculum)	1.3	1.4	1.4
Group 3 (not infected)	2.2	6.3	10.6

[1] **mean value derived from the ratio calculated for each animal**

Figure 5. 2 Comparison of thymidine uptake in popliteal lymph node cells cultured in medium alone and with IL-2



5.4 Discussion

The data show that the growth characteristics of lymphocytes derived from lymph nodes of rabbits with AHV-1-induced MCF are markedly different from that observed in cells derived from the two control groups. Since the cells used for these experiments were prepared in the same way as those in the phenotypic analysis described in chapter 4, it can be assumed, that all will be of similar phenotype. Variations in the responses of the three groups is not due to phenotypic differences. The results indicate that the short term cultures of lymphocytes from MCF affected rabbits are dysregulated.

The different culture conditions (medium alone, IL-2 and Con A) will be discussed separately.

5.4.1 Short term cultures of lymphocytes derived from MCF affected rabbits: *In vitro* hyperplasia?

The results show that freshly explanted lymphocytes derived from MCF-affected animals cultured in medium alone increased in number in the first 144 hours and that these cells incorporated up to five times more thymidine in the first 24 hours than the controls (Table 5.1 A and 5.2). The cells from uninoculated animals incorporated low levels of thymidine as would be expected and the results suggest that the lymphocytes derived from MCF-affected rabbits are activated. The strong staining with anti-PCNA of lymphocytes *in vivo* (Chapter 4.3.1 and 4.4.2), supports the hypothesis that the increased uptake by cells cultured from lymph nodes reflects the active multiplication *in vivo*.

The thymidine uptake by lymphocytes derived from animals infected with apathogenic inoculum cultured in medium alone increased over time (Figure 5.2B). Since cell culture medium was present in the inoculum, this reactivity may reflect an antigen specific response to proteins (e.g. FBS) in the cell culture medium. The pathogenic inoculum also contained these components, however there was no such increase in thymidine uptake by lymphocytes of MCF-affected rabbits. The difference between the group infected with pathogenic and apathogenic inoculum strongly suggests, that the lymphocyte populations derived from the latter were severely dysregulated.

Even though the thymidine uptake by lymphocytes derived from MCF- affected rabbits remained comparatively high with respect to the controls, it decreased over time (Figure 5.2 A). Since the thymidine incorporation was measured only for 20 hours prior to the point of examination and the large numbers of dead cells present in the cultures (Table 5.1 B), the cpm will not reflect the accurate multiplication rate of these cultures. Overall these data might reflect the hyperplasia and concomitant degeneration of the lymph nodes as observed *in vivo* during the terminal phase of the disease (Chapter 3.3.2).

However, AHV-1 protein expression and virus specific DNA initially detectable only in one to two cells per 10^6 cells of affected lymphoid organs of rabbits in the pyrexic stage of the disease, increases by a thousand fold after 48 hours in culture (Patel and Edington, 1981; Bridgen *et al.*, 1992). These findings indicate that as soon as AHV-1-containing lymphocyte populations are explanted and cultured, the balance between host and virus is profoundly altered and control mechanisms which inhibit viral expression are switched off. Because of these changes observed by others, the hypothesis that the thymidine uptake is directly reflecting the *in vivo* situation must be considered carefully.

5.4.2 Is IL-2 upregulated in lymphocytes derived from MCF-affected rabbits?

Exogenous IL-2 increased the multiplication of cultured cells in both experimental systems (Chapter 5.2.1 and 5.2.2) showing that hrIL-2 is a valid reagent to use in the rabbit system.

The thymidine uptake by cells derived from not inoculated rabbits (group 3) increased up to more than 10 times when stimulated with exogenous IL-2 (Table 5.2). This increase of the SI_{IL2} over time (Table 5.3) might indicate that IL-2Rβ^+ cells are present in resting lymphocyte populations and that exogenous IL-2 upregulated the multiplication by direct (activation of the internal pathways which results in cell multiplication) and indirect (via IL-2Rα chain upregulation and secondary cytokine response) mechanisms (as reviewed by Waldmann 1993).

In contrast, the ratio between medium- and IL-2-stimulated cells in rabbits infected with apathogenic inoculum (group 2) remained constant (Table 5.3) which suggests that physiologically functioning antigen-stimulated lymphocyte populations can be further stimulated by IL-2 at the concentration employed, but that the synergistic effect of exogenous IL-2 does not vary over time. This may indicate, that the control mechanism for IL-2R expression, important for the normal immune response, is not altered by the dose of IL-2 employed.

The preliminary experiment shows that exogenous IL-2 increases the viability of the cultured lymphocytes derived from MCF- affected rabbits (Table 5.1 B). These data were further confirmed by the observed increase of the SI_{IL2} which was similar to the ratio observed in not-inoculated animals. As discussed in 5.4.1, it seems apparent that the lymphocytes from MCF affected animals are already stimulated and it remains to be shown whether the increase of the SI_{IL2} is due to a further upregulation of IL-2R as observed in not-inoculated animals, or if the negative feed back mechanism which controls IL-2R expression is interrupted. However, if IL-2 were to be upregulated in an uncontrolled manner, by whatever mechanism, in lymphocyte populations from MCF-affected animals, as hypothesised at the beginning of this study, large amounts of IL-2 would already be available in the supernatant and exogenous IL-2 should not influence the viability of freshly explanted lymphocytes or the SI_{IL2}.

The importance of the species from which IL-2 is derived does not seem to influence the response of cells derived from MCF-affected animals, since recombinant bovine IL-2 (rbIL-2) titrated on one MCF-affected animal gave similar results to rhIL-2. Recombinant rabbit IL-2 was not available.

Experiments to block the thymidine uptake by lymphocytes derived from MCF-affected animals with anti-IL-2R antibodies were considered. Unfortunately the amount of available rabbit anti-CD25 was insufficient, and the bovine anti-IL-2R mAb, IL-A III, failed to block IL-2 dependent rabbit cell lines.

These findings show that lymphocytes derived from MCF-affected animals can be further stimulated by IL-2 and that it is unlikely that IL-2 is overexpressed in these lymphocyte population. Furthermore they underline the severe dysregulation already observed in cultures grown in medium alone.

5.4.3 Con A does not stimulate lymphocytes derived from MCF-affected animals

The lymphocytes from both control groups responded in the expected fashion to the stimulation by Con A. The decreased incorporation of thymidine in these cultures in the last 24 hours can probably be explained by the exhaustion of growth factors in the medium. In contrast, lymphocytes from MCF-affected animals reacted differently in that thymidine uptake in cultures supplemented with Con A was not significantly up-regulated. Furthermore, OHV-2 immortalised bovine cell lines tested for their responsiveness to Con A were found to be insensitive to this mitogen (Chapter 9). The lack of responsiveness to Con A could be explained by alteration of the T-cell receptor complex through which the mitogen normally exerts its function or by the production of an inhibitory factor. Preliminary experiments which tried to establish the presence of an inhibitory factor did not confirm this hypothesis (data not shown).
These findings confirmed the data of Wils *et al.,* (1978) who showed that lymphocytes derived from AHV-1 infected rabbits failed to respond to PHA and PPD from the day of the onset of pyrexia. Specific lymphocyte stimulation with either cell-associated viral or cell-free viral antigens was not detected in either challenged or control animals in the same set of experiments. The authors suggested that a soluble immunosuppressive factor present in the sera of affected animals inhibits the normal specific lymphocyte stimulation.
Similar observations have been reported for other herpesviruses, like *Herpesvirus saimiri* (HVS), *Bovine herpesvirus-1* (BHV-1) and *Suid herpesvirus-1* (SHV-1). In the case of HVS, tumour-bearing owl monkeys showed severe impairment of T-cell functions (Wallen *et al.,* 1975). This was manifested in a loss of responsiveness to the T-cell mitogens PHA and Con A. The authors suggested that this loss of T-cell responsiveness is mediated by a lymphocyte subpopulation with suppressor function. It has also been shown that lymphocytes from Con A-stimulated monkey tumour cells and tumour derived cell lines produced a soluble anti-proliferation factor which inhibited the mitogen response of isogeneic and allogeneic peripheral lymphocytes (Neubauer and Rabin, 1979, Neubauer *et al.,* 1980).

Furthermore, Carter *et al.,* (1989) showed that BHV-1 suppressed the Con A response of T-cell clones and IL-2 dependent cell lines. This inhibition was not due to lytic infection by BHV-1 and addition of exogenous IL-2 did not reverse this inhibition. He therefore hypothesised that latent BHV-1 influences the mechanism which is important for the cell activation by Con A. A similar situation was described by Williams (1990) who showed that SHV-1 reduced the mitogen response of normal porcine PBMC.

In conclusion, it has been shown that explanted lymphocytes derived from lymph nodes of MCF affected rabbits do not proliferate in response to Con A. This observation furthermore emphasises the hypothesis that these lymph node cells are severely dysregulated.

5.5 Summary

1. Freshly explanted lymphocytes derived from lymph nodes of rabbits clinically affected with AHV-1-induced MCF are actively multiplying.
2. IL-2 increases the viability and the thymidine uptake by lymphocytes derived from AHV-1 affected rabbits.
3. The SI_{IL2} increases over time in AHV-1 affected animals.
4. Con A does not stimulate lymphocytes derived from AHV-1 affected animals.
5. Lymph node cells explanted during the acute phase of MCF are severely dysregulated.

Chapter 6

Detection of IL-2 activity in supernatants derived from short term cultures of spleen and popliteal lymph nodes from MCF-affected rabbits

6.1 Introduction

Interleukin-2 (IL-2), or T-cell growth factor (TCGF) as it was called initially, has a crucial position in the initial steps of physiological immune responses as it induces clonal expansion of effector T-cells and also the activation of other lymphocytes (Chapter 1.3). No natural pathology involving hyperproduction of IL-2 has been described so far, administration of IL-2 to mice showed the generation of alloreactive cytolytic T cells and resident NK cells (Hefeneider *et al.*, 1983). High doses of IL-2 induced T-cell multiplication in lung, liver, spleen, kidney and mesenteric lymph node in mice. Intermediate multiplication could be seen in blood and CNS, whereas the thymus, intestines, and skin were not affected (Ettinghausen *et al.*, 1985). Moreover the parenteral administration of IL-2 increased serum levels of IFN γ and TNF α by induction of the corresponding genes in PBMC (Kasid *et al.*, 1989; Hirano *et al.*, 1990; Sarvetnick *et al.*, 1990). As discussed in previous chapters, MCF in rabbits is characterised by T-cell hyperplasia in lymphoid and non-lymphoid organs and it was therefore hypothesised that IL-2 could be responsible for the observed lesions. To establish if the lymphoid hyperplasia characteristic for MCF is due to virus induced hyperproduction of IL-2, the presence of IL-2 activity in supernatants of short term cultures of lymphocytes derived from MCF-affected and control rabbits was investigated.

Since no monoclonal antibody for rabbit IL-2 was available and the monoclonal antibody for rabbit IL-2R was only available in very small quantities, it was not feasible to develop an ELISA for the detection of IL-2. It was therefore decided to use a bioassay for IL-2 activity. This technique has also the advantage that only the bioactive form of the cytokine will be detected, whereas other immunochemical methods would reveal also non-bioactive IL-2. The bioassay used was based on that described by Gillis *et al.* (1978). The technique consists of assaying the proliferative response of a continuous murine tumour specific cytotoxic T-cell line (CTLL) by measuring the rate of thymidine incorporation. It seemed appropriate to use this assay for the detection of rabbit IL-2 activity, since IL-2 is not considered to be species-specific (Gillis *et al.* 1978; Denyer *et al.* 1987).

6.2 Material and Methods

6.2.1 Maintenance of CTLL

The CTLL cells were maintained at $1x10^5$ cells/ml with 10 IU/ml of hrIL-2 in 10 ml flasks. The cells were passaged and restimulated with hrIL-2 every four days. For the assay, cells cultured for at least four days were washed twice in WM (Chapter 2.4.1). For cryopreservation, the cells were harvested 24 hours after IL-2 stimulation and resuspended in freezing medium (Chapter 2.4.4). The cell line was kindly provided by Dr. E. Innes from the Moredun Research Institute.

6.2.2 Preparation of positive control for rabbit IL-2

Single cell suspensions from popliteal and mesenteric lymph nodes of normal rabbits were prepared as described in chapter (Chapter 2.4.6). Lymphocytes were washed, placed in 10 ml flasks at 2 x 10^6 cells/ml and incubated with 5 µg/ml Con A overnight. Supernatants from Con A cultures were harvested and aliquoted in 1.8 ml Eppendorf tubes.

To inactivate Con A which might have a stimulatory effect on the CTLL cells, previously autoclaved Sephadex G-10 (Pharmacia LKB, Biotechnology AB, Uppsala) was added generously at circa 1/5 of the volume of the supernatant. The preparation was rotated overnight at 4^0C and centrifuged at 2200 g in a microfuge. Supernatants were filtered (Millex-GV, 0.45 µm, Millipore SA, France) and stored at -20^0C.

6.2.3 Mouse Anti-interleukin-2 receptor/CD 25

The antibody against mouse IL-2Rα (CD 25, clone AMT-13), characterised by Diamantstein and Osawa (1984), Osawa and Diamantstein (1984) and Diamantstein *et al.* (1985) was purchased from Boehringer Mannheim Biochemical (Cat. No. 881 155). Osawa and Diamantstein (1986) showed that the clone AMT-13 inhibits IL-2 driven proliferation.

For control purpose, a cocktail of five monoclonal antibodies of different isotypes against Border Disease Virus (donated by D. Dean) was used.

6.2.4 Assay for IL-2 activity

The CTLL cells were dispensed at 5 x 10^3 cells/well in 50 µl on flat-bottomed 96 well plates and 50 µl of sample was added. Each test sample was set up in quadruplicate. The incorporation of thymidine was determined after a total incubation period of 48 hours as described in chapter (Chapter 2.4.7). Medium, hrIL-2 and supernatant from rabbit Con A blasts were used as controls for every plate.

6.2.5 Detection of IL-2 activity in supernatants from MCF affected and control animals

Nine rabbits were intravenously infected with AHV-1 infected rabbit cells (BJ 853 and BJ 919) and killed on the second day of febrile reaction. Two rabbits immunised intramuscularly with tissue culture propagated Rotavirus and two rabbits which had failed to react following inoculation with cells containing apathogenic AHV-1 were used as controls. Single cell suspensions of spleen and both popliteal lymph nodes were prepared (Chapter 2.4.6) and cultured at 2 x 10^6 cells/ml in 10 ml flasks. Aliquots of 400 µl of supernatant were removed at 12, 24, 36, 48 and 72 hours. The clarified supernatants were stored at -20^{0}C. Prior to use, supernatants were thawed quickly and centrifuged at 2200 g. IL-2 activity was then tested as described above (Chapter 6.2.4).

The positive control cultures on every plate contained media supplemented with 5 IU/ml hrIL-2 and the IL-2 activity of the sample could therefore be expressed in IU/ml by the formula:

$$\text{IL-2 activity in IU/ml} = \frac{5\left(\text{cpm}_{\text{sample}} - \text{cpm}_{\text{medium}}\right)}{\text{cpm}_{\text{IL2}} - \text{cpm}_{\text{medium}}}$$

where $\mathbf{cpm}_{\mathbf{sample}}$ was the cpm obtained with the test sample, $\mathbf{cpm}_{\mathbf{medium}}$ was the cpm obtained with medium alone and $\mathbf{cpm}_{\mathbf{IL2}}$ was the cpm obtained with rhIL-2. The statistical difference between the two groups was evaluated by ANOVA, whereas the decrease of IL-2 activity over time was analysed by regression.

In addition, eight samples from submandibular lymph nodes, five from appendix, one from liver and one derived from a well defined lymphoid cell accumulation in the kidney derived from different AHV-1 infected rabbits were examined.

6.2.6 Test for toxicity of supernatants

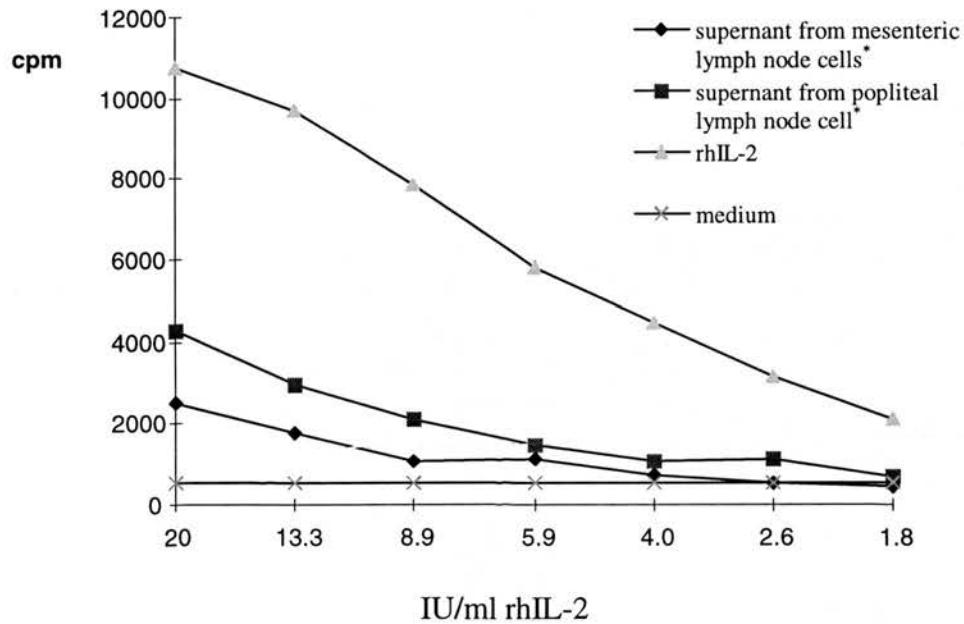
50 µl of test sample and 50 µl of 1/250 dilution of actinomycin D-mannitol were incubated for 24 hours over a monolayer of L929 TNFα sensitive and resistant cell lines. Cell lysis was determined after staining the cell monolayer with 0.5% Crystal violet in 20% methanol and determining the optical density on an ELISA microplate reader.

6.3 Results

6.3.1 Bioassay for the detection of rabbit IL-2 activity in supernatants

CTLL cells were sensitive to rabbit IL-2 activity derived from Con A blasts in a dose dependent manner (Figure 6.1). Medium supplemented with Con A and medium supplemented with Con A which was subsequently inactivated did not increase the proliferative response of the CTLL cell line.

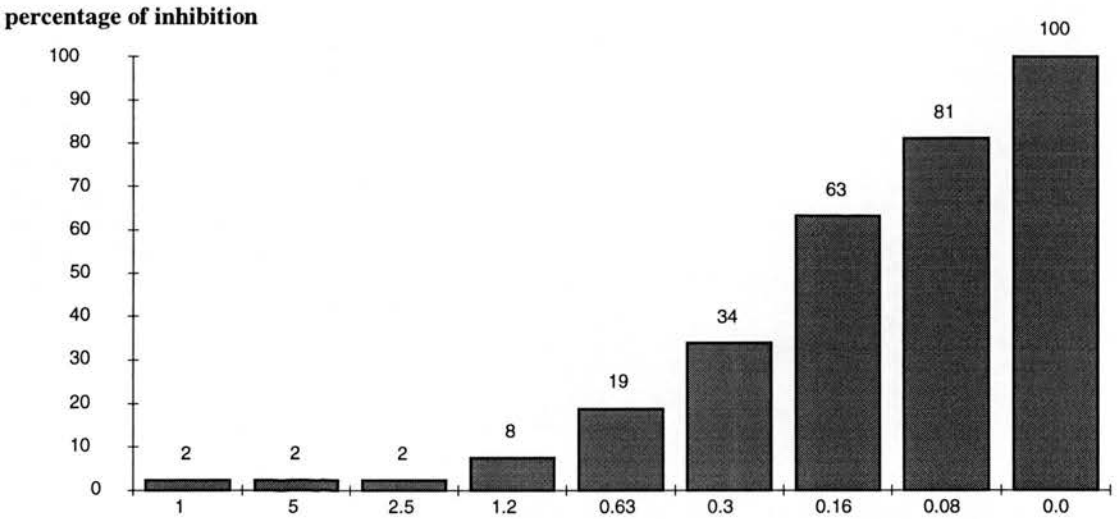
Figure 6. 1 Proliferative response of the CTLL cell line cultured with rhIL-2, medium alone and supernatants derived from mesenteric and popliteal lymph node cells stimulated with Con A for 18 hours



* 50µl supernatants from 18 hours Con A blasts in doubling dilutions

Specificity of the IL-2 activity was therefore tested by blocking multiplication with anti-mouse CD 25 antibody (Figure 6.2). The anti-CD25 antibody blocked the activity of rabbit IL-2 activity in a dose dependent manner, whereas the control antibody did not influence the incorporation rate of thymidine.

Figure 6. 2 Blocking of rabbit IL-2 activity with anti mouse IL-2Rα expressed in percentage of inhibition of the supernatant derived from Con A blasts



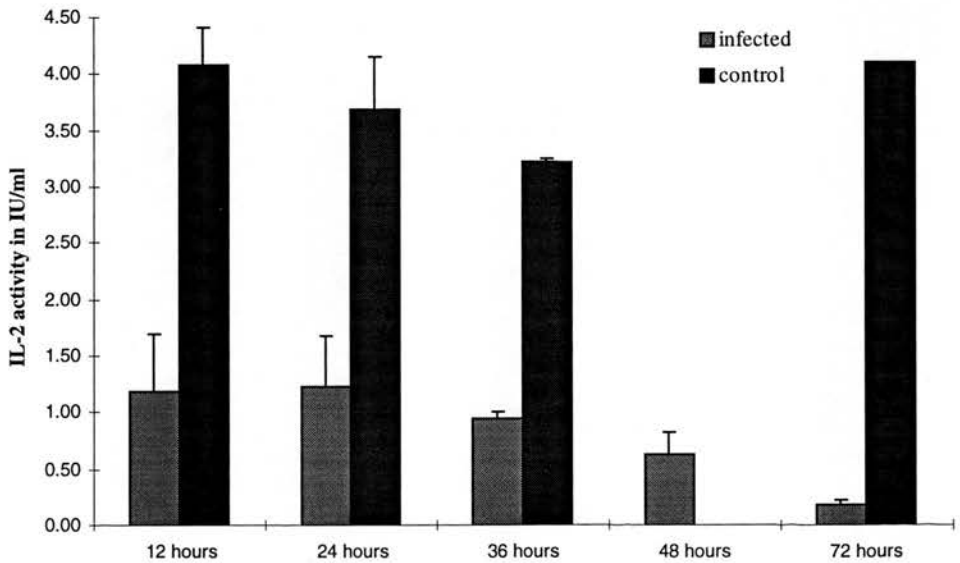
The reproducibility of the assay, using 5 IU of hrIL-2 as a reference, was tested by analysing the supernatant of rabbit Con A blasts on four different occasions. The results showed that the sample contained a mean value of 3.5 IU/ml of IL-2 with a standard deviation of 0.09. Samples which were tested repeatedly showed very little deviation (± 0.01).

6.3.2 IL-2 activity in cultures from MCF-affected and control animals

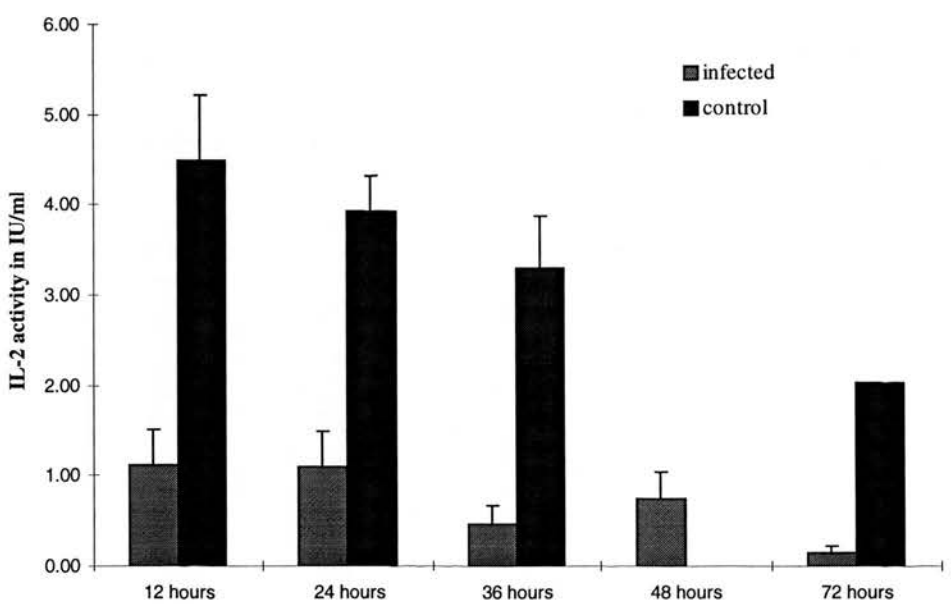
Supernatants of cells derived from spleen and popliteal lymph nodes of MCF-affected animals contained significantly less IL-2 activity than supernatants from control animals ($p < 0.001$) (Figure 6.3). The IL-2 activity decreased over time in supernatants derived from spleen ($p < 0.01$) and popliteal lymph nodes ($p < 0.05$) of AHV-1 infected animals. The IL-2 activity of supernatants derived from spleen of control animals decreased ($p < 0.05$) whereas the activity in supernatants derived from popliteal lymph nodes showed no significant decrease.

Figure 6. 3 Detection of IL-2 activity in supernatants of short term cultures derived from AHV-1 infected and control rabbits using the CTLL proliferation assay (mean value and standard error bar)

A- Supernatants derived from spleen cultures



B- Supernatants derived from popliteal lymph node cultures



Supernatants derived from short term culture of single cell suspensions from submandibular lymph node, appendix, liver and from a well defined lymphoid accumulation in the kidney all derived from MCF affected rabbits were all devoid of IL-2 activity.

None of the culture supernatants were cytotoxic for L929 cells.

6.4 Discussion

Significantly less IL-2 activity was detected in supernatants derived from short term cultures of spleen and popliteal lymph nodes from MCF-affected animals than from control cultures. This difference (Figure 6.3) could be a result of

1. inadequacy of the test employed
2. genuinely low levels of IL-2 synthesis by lymphoid cells derived from MCF-affected animals

or 3. large number of IL-2R$^+$ cells in MCF infected cultures which can deplete IL-2 from the culture supernatants

6.4.1 Inadequacy of the bioassay?

Gillis *et al.* (1978) showed, that stimulation of leukocytes with Con A induces the production of IL-2. Consequently, supernatants from rabbit Con A blasts were used to establish the validity of the assay. The IL-2 activity in supernatants examined from rabbit Con A blasts was comparable with other published findings. For example, Gillis *et al.* (1978) detected around 1 U/ml of TCGF in supernatants of mouse and rat Con A stimulated splenocytes and Tomai *et al.* (1989) detected between 2 and 5 IU/ml of IL-2 activity in supernatants from rabbit Con A stimulated splenocytes. Furthermore, it was possible to detect IL-2 activity in cultures derived from control animals, indicating that the test was valid.

The CTLL cell line is sensitive not only to IL-2, but also to other T-cell regulators such as IL-4 and IL-15 (Mosmann *et al.*, 1987; Thompson and Lotze, 1994). The activating factor for CTLL cells present in the supernatants derived from rabbits is likely to be IL-2, because its activity can be neutralised with anti-mouse CD 25 (Diamantstein and Osawa, 1986). This showed that the CTLL cells are activated by

binding of rabbit derived activating factor to the IL-2Rα which is specific for this cytokine (Chapter 1.3.2). The presence of other cytokines in supernatants of Con A stimulated rabbit cells could not be excluded because of lack of reagents. Further more, it did not seem necessary to differentiate IL-2 activity from the activity of other cytokines since the experiment showed that infected animals did have lower IL-2 activity than control animals (see below).

The presence of many dying cells (Chapter 5.4.1) suggests that toxic factors may have been present which could have compromised the multiplication of the CTLL cells. However, no toxicity against the L929 mouse cell line was detected in these supernatants. Therefore the presence of cytotoxic factors seems unlikely. Furthermore,

IL-2 activity was detected in supernatants of the cultures derived from MCF-affected animals during the first 24 hours of culture.

The assay was therefore considered a valuable and reproducible test for the detection of rabbit IL-2 activity.

6.4.2 MCF: Hypoproduction of IL-2 or high number of IL-2R$^+$ lymphocytes?

Since the explanted lymphocytes derived from **control rabbits** were probably stimulated by antigens in the culture medium (Chapter 5.4.1), it was expected to detect IL-2 activity in these supernatants. Some of the IL-2 is probably bound by the high affinity IL-2R complex which is similarly upregulated while any surplus could be detected in the supernatants (Chapter 1.3.2).

The low levels of IL-2 activity in supernatants from short term cultures of **MCF-affected rabbits** could either be due to IL-2 hypoproduction by these cells or to high levels of IL-2R$^+$ expression in the explanted cells. The two possibilities could not be tested since not enough monoclonal antibody was available to block the binding of IL-2 to IL-2R in the short term cultures.

The initial presence of IL-2 in supernatants of MCF-affected rabbits indicates that IL-2 producing cells are present *in vivo* during the acute phase of the disease and in the explanted lymphocyte populations. As suggested in chapter 4 and 5, pathological hyperplasia, humoral immune responses and secondary events due to cell destruction

probably overlap during the acute phase of the disease and therefore it is likely that IL-2 secreting cells are present in the lymph node and spleen. The decline of IL-2 activity with time in these supernatants could suggest that some of the explanted cells may express high levels of IL-2R and bind most of the endogenously produced IL-2, thus, IL-2 is no longer detectable in the supernatant.

The growth of explanted cells from MCF affected rabbits is considerably increased during the first 24 hours by exogenous hrIL-2 (chapter 5). Furthermore, it is possible to establish IL-2 dependent cell lines (chapter 1.2.6.) from MCF affected animals. These observations support the hypothesis that a dysregulated subpopulation of explanted lymphoid cells is effectively IL-2 sensitive and consequently expresses high levels of IL-2R.

For the further evaluation of the role of IL-2 in the pathogenesis of MCF *in vivo,* the presence of IL-2 message was investigated.

6.4.3 Summary

1. The CTLL cell line is a valid system for the detection of IL-2 activity in supernatants of rabbit lymphocytes.
2. Supernatants of short term cultures from MCF-affected rabbits contain significantly less IL-2 activity than supernatants from controls.
3. IL-2 activity in supernatants from MCF-affected rabbits decreased significantly over time.

Chapter 7

Molecular analysis of rabbit IL-2 mRNA and its application in the study of MCF-affected rabbits

7.1 Introduction

Human interleukin-2 (IL-2) was first characterised biochemically as a glycoprotein with a molecular weight of 15.5 kDa (Taniguchi *et al.*, 1983) and its cDNA was sequenced by Devos *et al.*, (1983) and Taniguchi *et al.*, (1983).

The possible role and significance of IL-2 in the pathogenesis of MCF has already been discussed in the previous chapters. To investigate whether IL-2 is transcribed during the acute phase of the disease in rabbits, it was necessary to develop a test which allowed the detection of IL-2 mRNA from tissue directly *ex vivo*. First attempts to amplify rabbit IL-2 mRNA using ovine IL-2 specific primers were unsuccessful. Since the sequence of rabbit IL-2 was not available in 1994 when the study was initiated, the most effective approach for the detection of IL-2 mRNA was to clone part of the rabbit IL-2 cDNA in order to develop a specific RT-PCR. At the start of this study, the IL-2 cDNA sequences from mouse (Kashima *et al.*, 1985), gibbon (Chen *et al.*, 1985), cattle (Ceretti *et al.*, 1986; Reeves *et al.*, 1986; Reeves *et al.*, 1987), rat (McKnight *et al.*, 1989), domestic sheep (Goodall *et al.*, 1990), pig (Goodall *et al.*, 1991), horse (Tavernor, 1992), domestic goat (Rimstad, 1993), cat (Cozzi *et al.*, 1993), manatee (Cashman, 1994) and red deer (Lockart, 1994) had been published (Table 7.2). These sequences were examined for highly conserved regions to design primers which could be used for the amplification of rabbit IL-2 cDNA by PCR. On the basis of the sequence obtained for rabbit IL-2, a RT-PCR followed by Southern blot was developed to evaluate the presence of IL-2 mRNA in spleen and popliteal lymph nodes of MCF-affected and control animals.

As with other inducible proteins, IL-2 expression can be regulated at various levels. Several functional regulatory sequences controlling transcription including binding sites for NFAT-1, NF-κB, AP-1 and octamer proteins have been identified within the IL-2 promoter region (Durand *et al.*, 1988; Shaw *et al.*, 1988; Hoyos *et al.*, 1989, Muegge *et al.*, 1989; Serfling *et al.*, 1989). In addition, IL-2 gene expression is also controlled at the post-transcriptional level involving instability of mRNA, apparently mediated through AU-rich sequence motifs present in the 3'end untranslated region of the IL-2 mRNA (Shaw and Kamen, 1986). However, unlike some other proteins,

no post-translational mechanisms of controlling IL-2 production have so far been found. Thus it may be safe to assume that the RNA detected by RT-PCR/Southern blot probably reflects the production of IL-2 protein.

7.2 Material and methods

7.2.1 Source of RNA

Single cell suspensions were prepared from the spleen of normal healthy rabbits and a normal healthy sheep as described previously (Chapter 2.4.6). These cells were then cultured at a concentration of 2 x 10^6 cells/ml in the presence of 5 µg/ml Con A. After 24 hours the cells were washed twice with WM and the RNA extracted as described previously (Chapter 2.5.1 to 2.5.3). The presence of RNA was verified by Northern gel analysis (Chapter 2.5.2).

Since the ultimate aim was to detect mRNA for IL-2 directly *ex vivo*, a rabbit was injected intramuscularly in the hind leg with ovalbumin (0.5 mg/ml) in incomplete Freund's adjuvant 120, 24 and 6 hours before it was euthanised. The spleen was removed, one half was snap-frozen in liquid nitrogen while the remainder was used to prepare a single cell suspension. For the single RT-PCR, 1 µg of total RNA was used.

7.2.2 Oligonucleotides used for the detection of IL-2 and actin

The primers for rabbit **IL-2** were designed on the basis of the sequence data extracted from the EMBL Database from 12 other species. Various primer sets, designed for bovine and ovine **actin,** were used for the detection of rabbit actin (AN: X60732 for α-smooth muscle actin; X60733 for β–non muscle actin). Their sequences and the sequences of the internal probe are shown in table 7.1.

7.2.3 Reverse transcriptase PCR (RT-PCR) and Southern blot

The RT-PCR was carried out as described previously (Chapter 2.5.4 and 2.5.5). For the development of the technique, the RT-step was performed with the specific IL-2

3' end primer (Chapter 7.2.2). For test samples, cDNA was synthesised with random primers (Chapter 2.5.4) and diluted 1:5 before proceeding to the PCR step with specific primers (Chapter 2.5.6 and 7.2.2). The presence of an IL-2 specific PCR product was than verified by Southern blot (Chapter 2.5.7 and 8) with hybridisation performed at 55^0C. When both IL-2 and actin primers were present in the PCR reaction (double PCR), the gel was blotted onto two membranes ('sandwich blot': one membrane on either side of the gel) to allow the hybridisation of the same product with two different probes.

7.2.4 Cloning of the PCR product

To clone the PCR-product, the TA Cloning™ System (Invitrogen Corporation, UK) was used. Briefly, the rabbit PCR- product was precipitated with 3 M sodium acetate and ethanol, in order to remove non incorporated nucleotides and primers, before being redissolved in distilled water. It was then ligated overnight at 12^0C to the pCR™ vector following the manufacturer's instructions. Competent *E. coli* cells were then transformed with the ligation products and plated on agar containing ampicillin at 50 µg/ml, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside). Recombinant clones were identified on the basis of their inability to convert the colourless substrate (X-gal) into a blue chromophore. After overnight incubation at 37^0C, 6 white colonies were isolated and grown in 10 ml L-broth for a further 18 hours. The plasmid was isolated, using the rapid alkaline extraction method (Birnboim, 1983). A series of buffers (Alk lysis I, II and III) which lyse and precipitate the cell debris while allowing the plasmid and the RNA to stay in the aqueous phase were used followed by treatment with RNase A to destroy the RNA. Plasmid DNA was precipitated with 100% ethanol and redissolved in 10 mM TRIS and 1 mM EDTA solution. The 6 TA-IL2 transformants were screened for the presence of an insert with the help of an enzyme digestion assay using EcoRI (Boehringer, Mannheim). EcoRI restriction sites are present on either side of the insertion region in the TA-vector.

Table 7. 1 Oligonucleotides used for the detection of IL-2 and actin

Specificity	Function	Code	Sequence	T_m	product size
IL-2	5'end	H0732	att gaa aca tct tca gtg tct a	58^0 C	206 bp
	3'end	H0733	cca ttt gtt cag aaa ttc tac a		
	probe	H3187	aga aga act caa acc tct gga gga agt gct taa ctt agc t	65^0 C	
Actin	5'end	492	tta caa cga gct gcg tgt gg	56^0C	825 bp
	3'end	521*	aga ctc gtc ata ctc ctg		
	5'end	522	atg gag aag atc tgg cac	52^0C	851 bp
	3'end	521*	aga ctc gtc ata ctc ctg		
	5'end	16	gag aag ctg tgc tac gtc gc	64^0C	262 bp
	3'end	17**	cca gac agc act gtg ttg gc		
	probe	M1866	ctc ctt gga gaa gag cta tga gct gcc cga tgg tca	65^0 C	
		M1867*	ttc atg atg gaa ttg aag gtg gtc tcg tgg atg cca		

T_m approximate melting temperature

* designed by Dr. C. McInnes, based on bovine actin by Degen *et al.* (1983) and his own analysis of ovine actin** designed by Dr. R. Collins, based on his own sequence analysis specific for bovine actin

7.2.5 Sequencing of rabbit IL-2

The sequencing reaction was performed with the T7 sequencing kit (Pharmacia) following the manufacturer's instructions. Two clones were selected to confirm the identity of the PCR product. The M13 forward and reverse primers were used to ascertain the sequence in both directions. Briefly, 8 µl of the plasmid miniprep were denatured at room temperature for 10 minutes by addition of 2 µl of 2M NaOH, and then precipitated by the addition of 3 µl of 3M sodium acetate, 7 µl of distilled water and 60 µl of 100% ethanol. Precipitated DNA was collected by centrifugation (16,000g/15 minutes), washed with 80% ethanol and redissolved in 10 µl of distilled water. Sequencing primers were annealed at 65^0 C for 3 minutes, and the mixture was cooled to $<35^0$C over 30 minutes. The labelling reaction (room temperature/3-5 minutes) with 0.37 MBq of ^{35}S was followed by the termination reaction (37^0C/5 minutes). The material was electrophoresed on a sequencing gel (6% polyacrylamide, 42 % (w/v) urea, 0.08 % APS, 0.04% TEMED in 1 x TBE). Two loadings were performed at an interval of 2 hours and a total running time of 5 hours. The gel was soaked afterwards in 10% glacial acetic acid, 10% methanol to remove the urea and dried. The results were visualised by autoradiography, analysed and compared with the other IL-2 sequences present in the database using the 'gcg-sequence analysis package' (Program manual for the Wisconsin Package, 1994).

7.2.6 Detection of IL-2 mRNA in spleen and popliteal lymph nodes of MCF affected and control animals

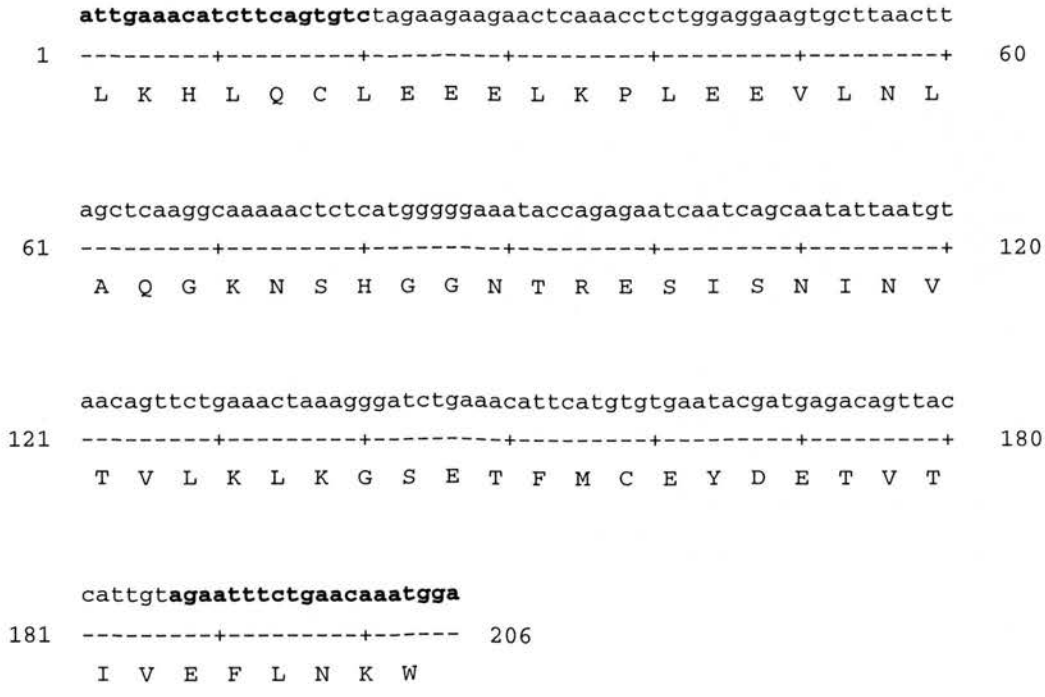
Seven rabbits were infected with pathogenic AHV-1 (Chapter 2.1.3), monitored and killed at the second day of febrile response (Chapter 2.1.1). For control purpose, eight hyperimmunised rabbits were used (Chapter 2.1.3). Single cell suspensions were prepared from spleen and popliteal lymph nodes. From the recovered cells, RNA was extracted and quantified as described previously (Chapter 2.5). For each RT-PCR reaction, 1 µg total RNA was used. RNA prepared from Con A blasts (Chapter 7.2.1) was used as a positive control.

7.3 Results and Discussion

7.3.1 Cloning and sequencing of rabbit IL-2

RT-PCR analysis of rabbit Con A stimulated lymphocytes, using the designed primers resulted in a product of comparable size to the product derived from ovine Con A stimulated lymphocytes, strongly suggesting, that the product obtained was rabbit IL-2. The sequence was determined in two independent clones in both directions. The comparison and analysis of the obtained sequence confirmed its identity as IL-2. The sequence with the predicted translation is shown in figure 7.1.

Figure 7. 1 Rabbit IL-2 nucleic acid* and predicted amino acid sequence



* primers are represented in bold

To investigate further the identity of this nucleic acid sequence (NAS) comparative analyses were done between the rabbit sequence and the sequences of 12 other species (Table 7.2).

Table 7. 2 Comparison of the rabbit IL-2 sequence obtained with the sequences from 12 other species

Species	Common name	AN	% AA	% NA
Suis scrofa	pig	X56750	89.7	82
Hylobates lar	gibbon	M11144	88.1	86
Homo sapiens	human	V00564	88.1	85
Equus caballus	horse	X69393	87.3	72
Felis catus	cat	L19402	85.3	84
Trichechus manatus	manatee	U09420	81.3	80
Rattus norvegicus	rat	M22899	77.9	70
Mus musculus	mouse	X01772	76.5	71
Bos taurus	cattle	M13204	75.0	76
Ovis aries	sheep	X53934	75.0	76
Capra hircus	goat	X76063	70.6	73
Cervus elaphus	deer	U14682	70.6	66

AN: Accession number in the DERC Daresbury Database

% NA: Percentage of *nucleic acid homology* given by the program FASTA

% AA: Percentage of *amino acid similarity* given by the program GAP

The FASTA analysis does an approximate alignment of the input NAS with all other NASs present in the database and displays the more closely related NASs. In this search only sequences resembling IL-2 were displayed. The multiple alignment of the NASs showed two significant gaps in the input NAS at position 183 and 200. When the rabbit NAS was translated into amino acid sequence (AAS), the gaps were found to correspond to single amino acids at the relative positions 58 and 64 of the

AAS alignment (Figure 7.2). Further evidence that the sequence obtained is rabbit IL-2, is the fact that cysteine in the relative position 6 and 61 are conserved (arrow in figure 7.2). These highly conserved cysteine residues form a disulphide bond in the mature protein that is required for the biological activity of IL-2 (Tsuji *et al.,* 1987; Brandhuber *et al.,* 1987).

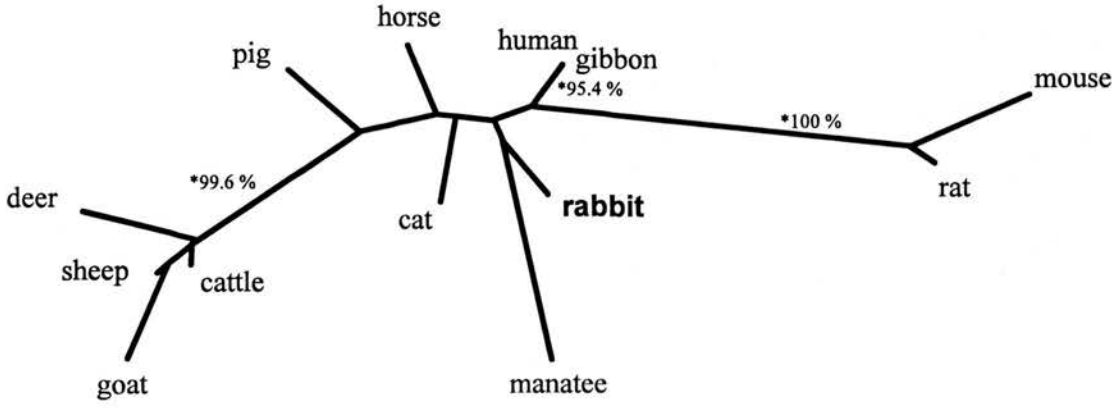
Figure 7. 2 Comparison of IL-2 amino acid sequence between rabbit and 12 other species

```
                  ↓    10            20            30            40            50
rabbit    LKHLQCLEEE LKPLEEVLN- AQGKNSHGGN TRE..... SISNINVTVL
pig       -------V-- --A--G---- G-S---DSA- IK-..... -MN-------
gibbon    ---------- ---------- --S--F-LRP .-D..... L------I--
human     ---------- ---------- --S--F-LRP .-D..... L------I--
horse     ---------- ------M-.. ...--FLSKD IK-..... LM--------
cat       -T-----V-- --------Y- --S--F-LNH IK-..... LM--------
manatee   -------V-- -----D---V -PS-Q..... ---..... L-------A-
rat       --------N- -GA-QR--D- T-S-SF-LED AGN..... F----R---V
mouse     --D-----D- -G--RH--D- T-S-SFQLED AEN..... F----R---V
cattle    ----K--L-- --L------- -PS--LNPRE IKD..... -MD--KRI--
sheep     ----K--L-- --L-----D- -PS--LNTRE IKD..... -MD--KRI--
goat      ----K--L-- --L--D--D- -PS--RNTRE IKD..... YMASLKGI--
deer      ----N--L-- --L--D--S- SPS--LNPKE IKDSMDEIKD LMD--KRI--

                     60 ↓          70       77
rabbit    KLKGSET.FM CEY.DETVTI VEFLNKW
pig       E------S-K ---D-----A -------
gibbon    E------T-- ---A---A-- -----R-
human     E------T-- ---A---A-- -----R-
horse     G------R-T ---D---G-- -------
cat       -------R-T -N-D---A-- -------
manatee   E-Q--K-P-- ---D-KAA-- E----N-
rat       ------NK-E -QFD--PA-V ----RR-
mouse     -----DNT-E -QFD--SA-V -D--RR-
cattle    E-Q----R-T ---D-A--NA -------
sheep     E-Q----R-T ---D-A--KA -------
goat      E-Q----R-T ---D-A--KA ---Q---
deer      E-Q----S-K ---DAA--KA -------
```

A phylogenetic tree was constructed from the amino acid sequence of rabbit IL-2 and IL-2 from 12 other species by applying the neighbor-joining method (Saitou and Nei, 1987) to pairwise distances that had been corrected for multiple hits by the method of Kimura (1983). Statistical testing of the tree topology was done using the bootstrap method based on 1000 trials (Hedges, 1992). The bootstrap results are summarised as the percentage of trials supporting a given node. It is interesting to note that even though IL-2 is known to be a highly conserved locus in respect to other cytokines, a distinct pattern can be recognised: Ruminantia, Primates and Rodentia have statistically significant clusters, with the other animals most closely related to the Primates (Figure 7.3).

Figure 7. 3 Phylogenetic tree for IL-2 based on the partial amino acid sequences of 13 species



* bootstrap results given in percent
(only values over 95% are reported)

NB. Only the cluster for mouse/rat and ruminants was statistically significant

7.3.2 Development of a RT-PCR/Southern blot for the detection of IL-2 mRNA in rabbit tissues

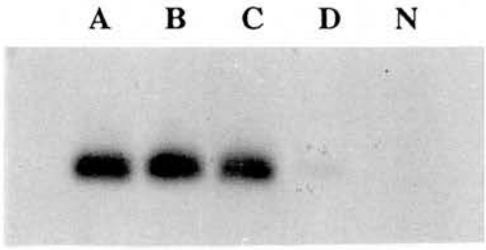
The rabbit IL-2 cDNA sequence was used to establish a sensitive and precise technique for the detection of rabbit IL-2 mRNA. An internal probe was designed from the cDNA sequence to be used for confirming the identity of the PCR products by southern blotting. The probe was designed, so it could also be used for the detection of bovine IL-2 cDNA (Figure 7.4)

Figure 7. 4 Internal probe for the detection of IL-2 mRNA from rabbit and bovine tissue

```
rabbit   aga aga act caa acc tct gga gga agt gct taa ctt agc t
         ||| ||| ||| ||| ||  |||  ||  ||| ||| |||  ||  ||  ||| |
bovine   aga aga act caa act tct aga gga agt gct aaa ttt agc t
```

To establish the validity of the method, Con A blasts from seven rabbits were examined by RT-PCR/Southern blot. Figure 7.6 verifies the specificity of the probe as well as establishing the validity of the technique using outbred rabbits. The sensitivity was tested by titration of total RNA derived from Con A blasts. It was possible to detect a signal with only 1 μg of total RNA. Considering that the mRNA is only a very small percentage of the total RNA extracted and that the specific cytokine DNA will only be a small fraction of this, the detectable amount of specific cytokine RNA is estimated to be in the fg/pg range (Figure 7.5 and 7.6).

Figure 7. 5 Serial dilution of rabbit RNA derived from Con A blasts of a control rabbit amplified by RT-PCR/Southern blot specific for rabbit IL-2



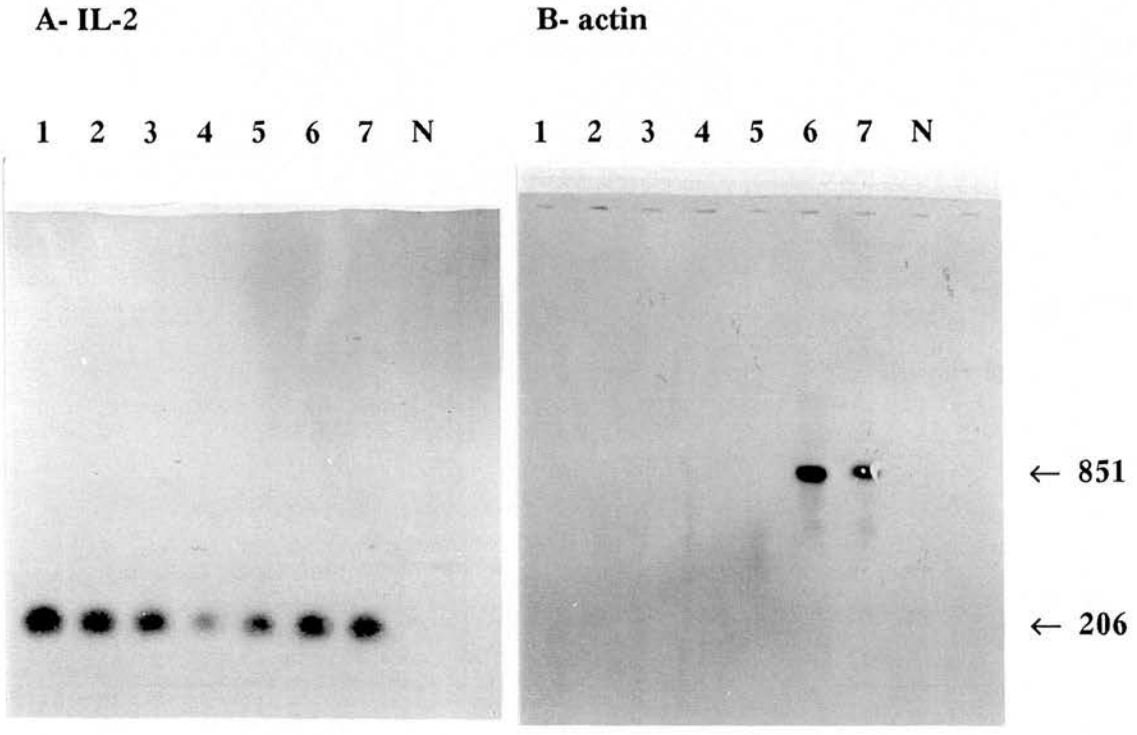
A 1 μg total RNA derived from Con A blasts of a control rabbit

B -D serial dilution of the same RNA (1:10) **N** negative control

In order to provide a positive internal control for every sample, to validate the quality of the RNA preparation particularly in negative samples, actin was chosen based on the assumption that α, β or γ actin would be transcribed in every cell. A double PCR was therefore attempted for actin and IL-2 RNA.

Different primer pairs and annealing temperatures were tested unsuccessfully even though no large mismatches between the primers and the published actin sequences for rabbits and other species were found. The primer pair 521/522, gave good results when used in single reactions, but did not give reproducible results when coupled with the RT-PCR for the detection of IL-2. As can be seen in figure 7.6 A and B, all samples were positive for rabbit IL-2, indicating that the RNA was not degraded and that the reaction was working. However, the RT-PCR/southern blotting for actin worked only in two samples out of seven. Personal discussion with various experts in molecular biology indicated that enzymes involved in cellular respiration would have been better as a positive control. Since IL-2 mRNA was detected in all samples examined (Chapter 7.3.3), no further attempts were made to optimise the technique.

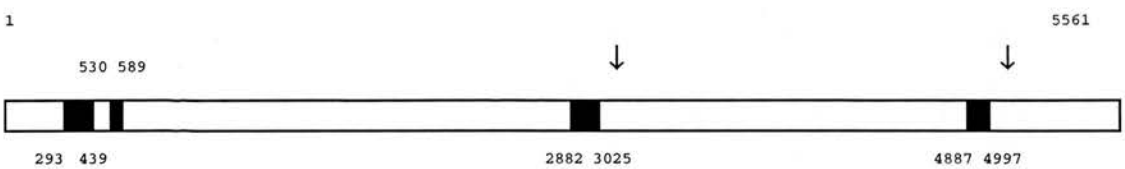
Figure 7. 6 Double RT-PCR followed by Southern blot from the same gel for the detection of IL-2 (A) and actin (B) mRNA on RNA derived from splenocytes stimulated with Con A. Note: insufficient detection of actin RNA in presence of IL-2 RNA



1-7 RNA derived from Con A blasts of seven outbred rabbits **N** negative control

To discriminate between RT-PCR arising from mRNA as opposed to that from contaminating genomic DNA, the primers had been designed from two different exons. From a study of the human IL-2 gene (Holbrook *et al.,* 1984), it had been predicted that the primers would anneal at positions 2890 and 4937 of the complete gene. The probe employed in this study was predicted to anneal at position 2914 to 2943 (Figure 7.7). Thus it was assumed that a PCR product obtained by amplifying the gene would be of much larger size and therefore easily detectable. Conservation of the intron/exon boundaries of cytokine genes between species has been shown for both the IL-3 gene (McInnes *et al.,* 1994) and the IFNγ gene (Schmidt and Seyfert, 1995).

Figure 7. 7 Organisation of the human IL-2 gene (AN: K02056)



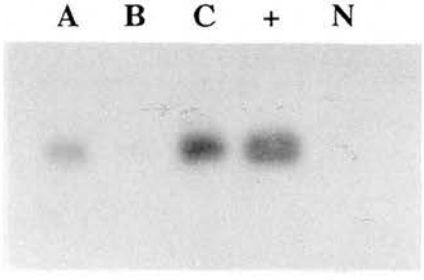
black: coding region

white: non coding region

↓ approximate annealing position of the primers

Since the aim of the study was to detect IL-2 mRNA directly *ex vivo*, two different methods of preparing the initial material were compared. The signal derived from snap-frozen material was less intense than the signal derived from the single cell suspension (Figure 7.8). This could be explained by the fact that more non specific RNA, such as that derived from connective tissue, is present in the snap-frozen material. It was therefore decided that single cell suspensions would be a better starting material for the detection of IL-2 mRNA than snap-frozen material.

Figure 7. 8 Comparison of the detection of IL-2 specific mRNA derived from snap-frozen spleen and single cell suspension derived from spleen of a hyperimmunised rabbit



A single cell suspension derived from the spleen

B snap-frozen spleen

C splenocytes from the same rabbit stimulated for 24 hours with Con A

+ positive control

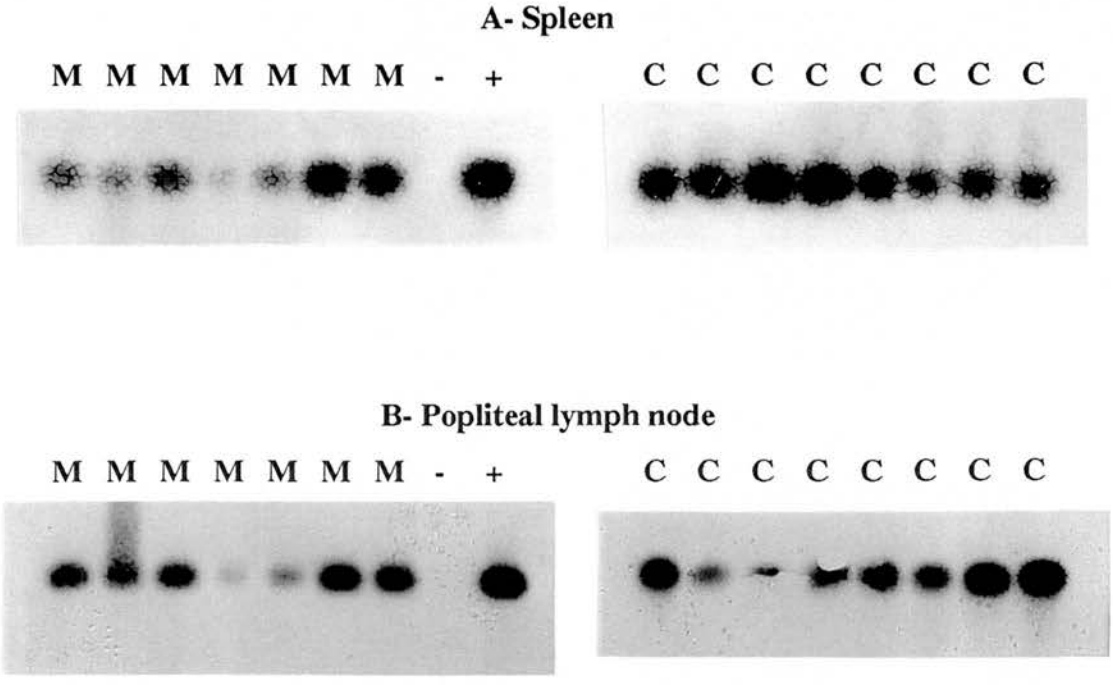
N negative control

7.3.3 Detection of mRNA specific for IL-2 in spleen and lymph nodes of MCF affected and control rabbits

The established assay was used to evaluate the transcription of this cytokine directly *ex vivo* during the acute phase of the disease. The method was applied for the detection of IL-2 mRNA from spleen and lymph nodes of MCF affected (malignant hyperplasia) and antigen stimulated control rabbits (benign hyperplasia). The seven rabbits infected with AHV-1 strain C500 developed disease with a mean incubation period of 12.4 days (SEM = 0.9) and the pathological changes were consistent with the diagnosis of MCF, whereas in the eight control rabbits the only lesions detectable were slight hyperplasia of the popliteal lymph nodes and fibrosis of the site of inoculum.

IL-2 mRNA was detected in both spleen and popliteal lymph nodes derived from all MCF-affected and control rabbits (Figure 7.9). Since the amount of RNA taken for each RT-PCR was standardised at approximately 1 µg per reaction and the bands obtained were of comparable intensity in both groups, it could be suggested, that IL-2 is transcribed in a similar amount in spleen and popliteal lymph nodes in both groups. As described in the introduction, this result should reflect the actual protein expressed.

Figure 7. 9 Detection of IL-2 mRNA by RT-PCR/Southern blot in spleen (A) and popliteal lymph nodes (B) from MCF affected and control animals



M MCF-affected rabbits - negative control + positive control **C** control rabbits

As the RNA was extracted from the whole lymphoid organs, containing a variety of lymphocytes, it was expected that lymph nodes and spleens of the control animals were activated and therefore contained IL-2 transcribing cells. In the MCF-affected rabbits the situation is more complicated, because it is not possible to distinguish between the primary and secondary events. The T-cell areas are certainly hyperplastic, but apoptosis and haemorrhages are also features of the disease, which will influence the cytokine profile. However, if AHV-1 does induce a hyperproduction of this cytokine, as hypothesised at the beginning of the study, the expected intensity of the bands would be much stronger in tissues from MCF affected rabbits compared to controls. Whereas, if IL-2 production was down regulated, as suggested from the data obtained by the detection of bioactivity in supernatants of short term cultures, no IL-2 message would have been detected (Chapter 6). In the general discussion (Chapter 10), the role of IL-2 in relationship to the pathogenesis will be discussed in detail.

From the data presented, it can be concluded that IL-2 is transcribed in a comparable amount in MCF-affected and control animals, independently of whether the hyperplasia is malignant (induced by AHV-1) or benign (induced by presentation of antigens), but it can not be established if IL-2 is induced as a primary or a secondary event.

7.4 Summary

1. Rabbit IL-2 mRNA was partially sequenced and the sequence was submitted on the 02.09.94 to EMBL/GenBank/DDBJ databases (AN Z36904).
2. The RT-PCR/Southern blot developed for the detection of IL-2 mRNA from a variety of samples is specific and sensitive.
3. IL-2 is transcribed to a comparable extent in MCF-affected and control animals.

Chapter 8

Pathology and short term cultures of lymph node cells derived from cattle affected with SA-MCF

8.1 Introduction

MCF has been described in rabbits under experimental conditions (Chapter 1, 3-7), but has never been identified in free living rabbits (Heuschele, 1980) and probably does not present itself as a naturally occurring disease. The adaptation of AHV-1 (Daubney and Hudson, 1936), OHV-2 (Buxton and Reid, 1980; Westbury and Denholm, 1982) and HipHV-1 (Reid and Bridgen, 1991) to rabbits has, therefore, to be considered a laboratory model. Experimental infection of rabbits with these pathological agents is however a useful model for investigating the pathogenesis of MCF for comparison with the naturally susceptible host.

Under experimental conditions, WA-MCF can be readily transmitted from affected cattle and normal wildebeest to cattle using fresh cell suspensions (Plowright, 1965b). In contrast, experimental transmission of SA-MCF from diseased cattle (*Bos taurus*) to cattle has been achieved only irregularly (Goetze and Liess, 1930, Magnusson, 1940; Blood *et al.,* 1961; Pierson *et al.,* 1974, Selman *et al.,* 1978; Reid *et al.,* 1986). However transmission of SA-MCF with white blood cells from affected red deer (*Cervus elaphus*) and Pere David's deer (*Elaphus davidianus*) to red deer, Pere David's deer, Rusa deer (*C. timorensis*) and roe deer (*C. capreolus*) is readily achieved (Huck *et al.,* 1961; Buxton and Reid, 1980, Reid *et al.,* 1986; Westerbury and Denholm, 1982; Oliver *et al.,* 1983, 1984).

Since it is not always possible to transmit MCF to cattle under laboratory conditions and the incubation period is relatively long and unpredictable (1.2.3.1), the source of material for this part of the study was naturally occurring cases of SA-MCF in cattle examined during this period and cell lines prepared as previously described by Reid *et al.,* (1983). In this chapter, the pathology of SA-MCF in naturally affected cattle and the characteristics of short term cultures of lymph node derived cells are described. The characterisation of the established cell lines from these animals will be described in chapter 9.

8.2 Materials and Methods

8.2.1 Animals

Fifteen cattle with clinical signs of MCF were referred through Scottish Agricultural Colleges Veterinary Services (SACVS) and private veterinary practitioners to the Moredun Research Institute during the period of this study. The animals were from various breeds, both sexes and apart from one female (5 years old) were aged between 6 to 12 months. A full post mortem examination was carried out as soon as possible after the animal arrived (Chapter 2.2). The confirmation of the clinical diagnosis was based on pathological changes and further confirmed by PCR and IIF (Chapter 1.2.4).

All materials used in these and the following experiments were derived from these animals.

8.2.2 Diagnostic PCR for OHV-2

DNA was extracted from lymph node cells (Chapter 2.5.5). The nested PCR specific for OHV-2 was performed as described by Baxter *et al.,* (1993). The total reaction volume was in 50 µl and contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01%v/v gelatine, 10% v/v DMSO, 100 µM dATP, dCTP and dTTP, 2 U *Taq* DNA polymerase and 1.0 µM of each primer. The preparation was heated at 99^0C for 3 min after which dNTP and the enzyme mix were added. This was followed by 25 cycles of 94^0C for 20 sec., 60^0C for 30 sec. and 72^0C for 30 sec. An aliquot of the primary amplification product (primer: 556/775) was transferred to a new reaction mixture and amplified under the same conditions with the second primer pair (primer: 556/555) (table 8.1). The final extensions step was at 73^0C for 5 min.

Table 8. 1 Primers specific for OHV-2

Primer	sequence (5' → 3'end)	product size
556	agt ctg ggt ata tga atc cag atg gct ctc	
755	aag ata agc acc agt tat gca tct gat aaa	422 bp
555	ttc tgg ggt agt ggc gag cga agg ctt c	238 bp

8.2.3 Indirect Immunofluorescence (IIF) for the detection of antibodies to AHV-1

Confluent monolayers of bovine turbinate primary cell cultures grown on glass cover slips were infected with AHV-1 (reference strain WC11) or BHV-4 (reference strain Movar 33/63) and fixed with acetone when cytopathic effects were detected generally five days after infection as previously described (Plowright, 1981).

For the diagnostic test, the fixed monolayers were incubated with heat-inactivated horse serum (1:20 in PBS) for 30 min at 37^0 C, washed in PBS and coated with test serum (1:10 in PBS). A known positive and a known negative serum were included in each test. The preparations were incubated for a further 90 min at 37^0 C. Fluorescein labelled donkey anti-sheep IgG polyclonal serum (known to be cross reactive with cattle serum, Scottish Antibody Production Unit, SAPU) was added and allowed to react for 30 min. Between each incubation, the slides were washed twice with PBS for 10 min. The preparations were mounted with 10% glycerine in PBS on each slide and examined on a Leitz fluorescent microscope.

8.2.4 Cell culture

Mesenteric, retropharyngeal, prescapular and prefemoral lymph nodes from four SA-MCF affected animals (BJ 971, BJ 1004, BJ 1044, BJ 1187) were removed and single cell suspensions were prepared (Chapter 2.4.6). As a control, lymph node cells from two cattle not affected with MCF were used. The first animal was a calf submitted as a case of MCF, but which was subsequently diagnosed as suffering of mucosal disease (serological and virological examination was kindly carried out by Dr. P. Nettleton). The second control calf was vaccinated against *Bovine herpesvirus-1* (BHV-1) and boosted after 21 days. The animals was subsequently challenged on day 42 and 120 with 10^8 pfu BHV-1 strain Iowa and killed 5 days after the last challenge. Pathological as well as the virological examination (histology, serology and PCR) of the two animals revealed no evidence of MCF.

The cells derived from this animals were cultured in medium alone or medium supplemented with 150 U/ml hrIL-2 or 5 µg/ml Con A at a concentration of 2 x 10^5 cells/well. This concentration was based on the experiments performed with material

from rabbits (Chapter 5). Multiplication of the leukocytes was determined by incorporation of ^{3}H-thymidine at 24, 48 and 72 hours (Chapter 2.4.7). Results were expressed either simply as cpm or as a stimulation index (SI_{IL2}) as described in chapter 5.2.2. Results were evaluated statistically using four way ANOVA.

In addition, retropharyngeal lymph node cells from one case (BJ 1004) were incubated with a mAb against bovine CD25 (IL-AIII) with or without stimulation by 300 U/ml IL-2R. The mAb IL-AIII neutralises IL-2 activity *in vitro* (Naessens *et al.*, 1992). As control antibody, a cocktail of five mAb of different isotypes against border disease virus was kindly provided by D. Dean (MRI, UK). The inhibition by mAb IL-AIII was expressed as an inhibition index (II_{CD25}):

$$II_{CD25} = \frac{cpm_{CD25}}{cpm_{control}}$$

where $\mathbf{cpm_{CD25}}$ is the thymidine uptake of the single dilution of anti-bovCD25 and $\mathbf{cpm_{control}}$ is the thymidine uptake of the respective control (medium alone or IL-2 supplemented). Results were evaluated statistically using Student's T-test.

8.3 Results

8.3.1 Pathology, PCR and IIF

The diagnosis of MCF was confirmed on pathological, serological and molecular biological basis in all but one animal. All animals referred showed pyrexia, enlargement of palpable lymph nodes, oral, nasal and ocular discharge of various characteristics from mucoid to thick catarrhal stringed with blood. Most animals manifested clinical signs of the head and eye form of MCF with bilateral oedema of the cornea and nervous symptomatology (Table 8.2). The pure intestinal disease, characterised mainly by diarrhoea, was observed in only three animals, whereas one animal showed signs of cutaneous MCF. In all cases, the disease had been suspected by the attending veterinary surgeon for at least 3 days, indicating a subacute to chronic course.

One animal (BJ 1008) with the typical clinical signs of intestinal MCF was diagnosed as suffering from mucosal disease on histological grounds (no changes in the CNS and no lymphoid cell accumulations) and by isolation of cytopathic BVDV.

At post mortem examination of all the MCF-affected animals, the respective clinical signs (eye or intestinal lesions) were confirmed (Table 8.2). The retropharyngeal, prefemoral, prescapular and mesenteric lymph nodes were enlarged to a varying degree (Table 8.2). The cortex of the lymph nodes appeared milky and focal necrosis and haemorrhages were regularly present. Congestion of the meninges and leucocytosis in the CNS was present in all animals. Ecchymosis and petechiae in the urinary and gall bladders were frequently present. The liver, though often enlarged, was not always affected. In four animals, the capsule of the kidney was easily removed and white foci of around one millimetre in diameter was found on the surface (Figure 8.1). Emphysema and atelectasis were often observed in the apical lobes of the lung. Haemorrhages were common in the trachea and larynx.

At histological examination, affected lymph nodes showed hyperplasia of the paracortex and deficiency of follicles, often associated with necrosis and haemorrhages. The spleen was always depleted. Lymphoid cell accumulations were observed in liver, kidney, urinary and gall bladders and various glands such as the pancreas and the salivary and lachrymal glands (Table 8.2). Segmental vasculitis in various organs was found in all but two animals (Figure 8.3). Focal paracheratotic hypercheratosis associated with lymphoid cell accumulations was found in the pregastric tract of all animals (Figure 8.2). In five animals, skin changes of the same character as that observed in the pregastric tract was observed. Oedema of the cornea was confirmed by histological examination and was always associated with lymphoid cell accumulations in the scleral plexus. Vascular cuffing in the CNS was detected in all MCF-affected animals, although to a variable degree. In the medullae oblongata and the medullae spinalis, Wallerian degeneration was a common finding.

These pathological findings were consistent with the diagnosis of MCF. For all these animals, the diagnosis was confirmed by OHV-2 specific PCR on lymph node cells and by the detection of antibodies to AHV-1 by IIF and (Chapter 1.2.4).

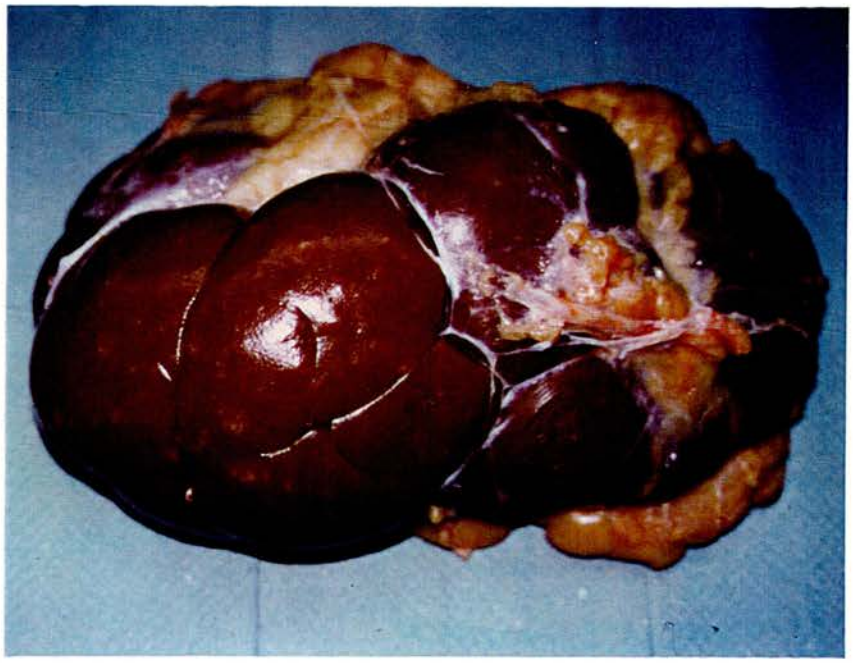
Table 8. 2 Pathological changes found in cattle affected with SA-MCF referred to the Moredun Research Institute

Case no	CF	CNS	RF[1]	PS[1]	PF[1]	M[1]	SV	skin*	PG	liver*	gBl*	kidney*	uBl*	GL*	PCR	IIF	CL
BJ 1196	h/e	c +++ wd	+	++	+	n	yes	n	pHC	+++	+++	+	++	ne	+	+	yes
BJ 1187	h/e intest	c + wd	++	++	n	++	yes	+	pHC	+	+++	+++	++	+	+	+	yes
BJ 1173	intest	c ++, wd	++	++	++	+	yes	+	pHC	++	+++	++	+++	+	+	+	yes
BJ 1104	skin	c +, wd	+++	+++	+	+++	yes	+++	pHC	n	++	n	+	+	+	+	yes
BJ 1044	h/e intest	c ++ wd	+++	+++	++	+	yes	ne	pHC	+	+++	+++	+++	n	+	+	yes
BJ 1035	h/e	c +, wd	+++	++	+	+	no	ne	pHC	+	+++	+++	+++	ne	+	+	yes
BJ 1004	intest h/e	wd	+++	+	n	+++	no	ne	n	n	+++	++	+++	ne	+	+	yes
BJ 971	h/e	c ++, wd	+	+	+	n	yes	+++	pHC	n	++	++	++	+	+	+	yes
BJ 934	h/e intest	c +, wd	++	++	++	n	yes	+	pHC	++	+++	+++	+++	+	+	+	yes
BJ 924	h/e	c ++, wd	++	+	++	n	yes	ne	pHC	n	++	++	+	+	+	+	yes
BJ 913	h/e	c +, wd	++	+	+	n	yes	ne	pHC	+	++	++	+++	+	+	+	yes
BJ 903	intest	ne	ne	++	ne	n	yes	ne	pHC	++	+	n	+++	ne	+	+	ne
BJ 889	h/e	c +, wd	ne	ne	++	+	yes	ne	pHC	+	++	++	++	+	+	+	yes
BJ 884	h/e	c +++, wd	ne	++	+++	n	yes	ne	pHC	+	+++	+	++	ne	+	+	yes

c cuffing; **CF** clinical form; **CL** cells grown from at least one lymph node for at least 2 months; **CNS** central nervous system; **gBL** gall bladder; **GL** glands such as pancreas, adrenal, salivary and lachrymal gland, **h/e** head and eye form; **intest** intestinal form; **M** mesenteric lymph node; **n** negative; **ne** not examined; **PF** prefemoral lymph nodes; **PG** pregastric tract; **pHC** paracheratotic hypercheratosis; [1] hyperplasia of; **PS** prescapular lymph node; **RF** retropharyngeal lymph node; **skin** skin form; **SV** segmental vasculitis in at least one organ; **uBl** urinary bladder; **wd** Wallerian degeneration; * presence of lymphoid cell accumulations only; + present, positive or increased number of lymphocytes, ++ marked increase of size of lymph node or marked increase lymphocytes in more foci, +++ large number of lymphocyte accumulations associated with necrosis and haemorrhages

Figure 8. 1 Typical lesions in the kidney of an MCF-affected animal (BJ 1004).

A - Post-mortem examination. Note the white foci.



B- Histological examination. Note interstitial lymphoid cell accumulation comparable to the lesions seen in the rabbit (Figure 4.8)

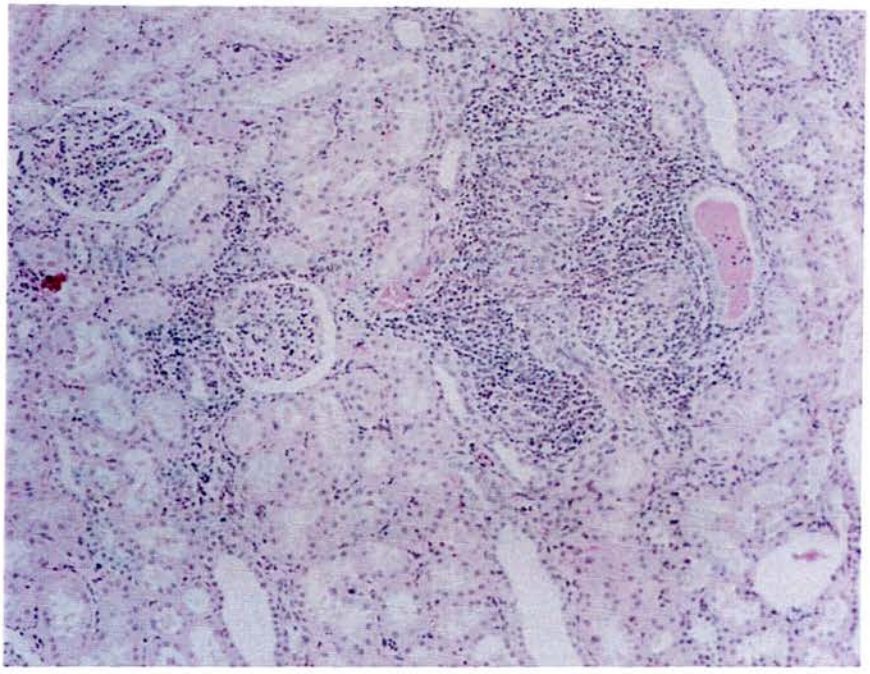


Figure 8. 2 Lymphoid cell accumulation associated with paracheratotic hypercheratosis in the reticulum of animal BJ 1035.

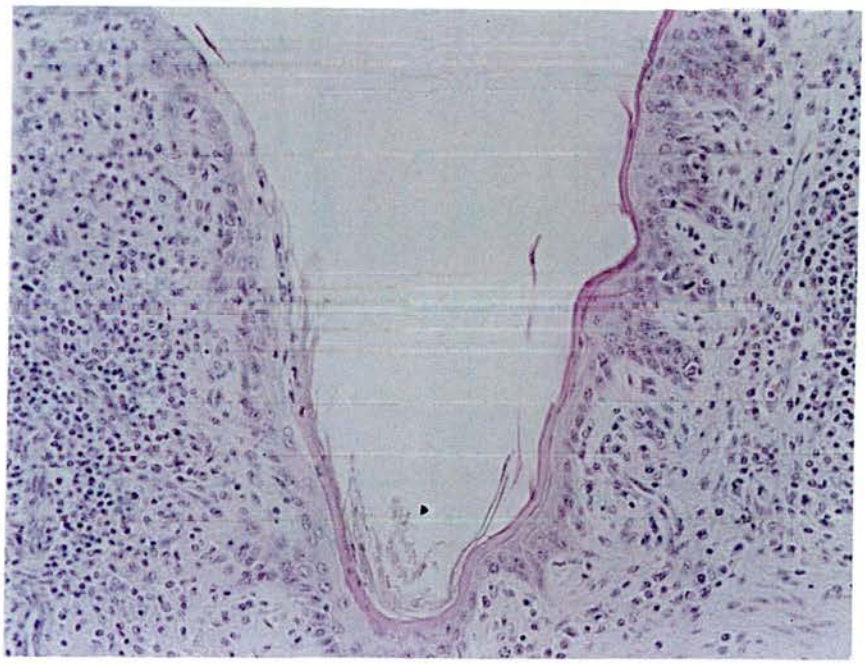
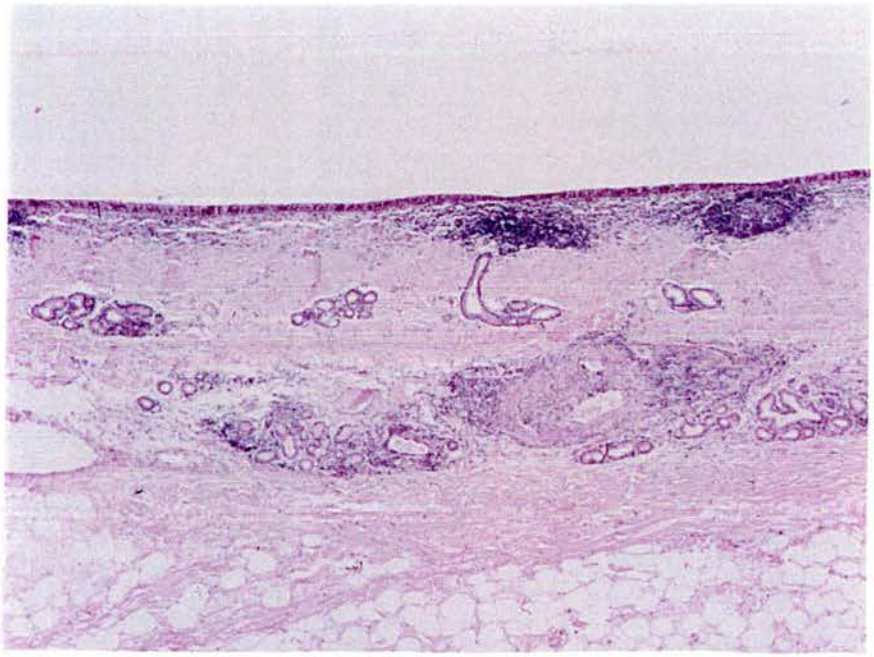


Figure 8. 3 Trachea of animal BJ 971. Note arteritis and lymphoid cell accumulations.



8.3.2 Short term cultures of leukocytes derived from lymph nodes from SA-MCF affected cattle

No significant difference in thymidine uptake by lymph node cells was observed between MCF-affected and control animals (Figure 8.4).

The short term cultures of lymph node cells of SA-MCF affected cattle showed that culture conditions and time had a significant ($p < 0.001$) effect on thymidine uptake whereas the derivation of the cells (animal and type of lymph node) did not (Table 8.3). The results can, therefore, be summarised as follows: -

Thymidine uptake by all lymph node cell cultures in **medium alone** increased significantly ($p < 0.01$) over time from a mean value of 2785 cpm (sem 624) at 24 hours, to 8299 cpm (sem 1611) at 72 hours. **Exogenous IL-2** enhanced thymidine uptake significantly ($p < 0.001$) from 3725 cpm (sem 700) at 24 hours to 20,166 cpm (sem 1270) at 72 hours. Lymph node cells cultured with **Con A** had a significantly ($p < 0.01$) higher thymidine uptake than the cultures with medium alone or IL-2 over the first 48 hours (Figure 8.4).

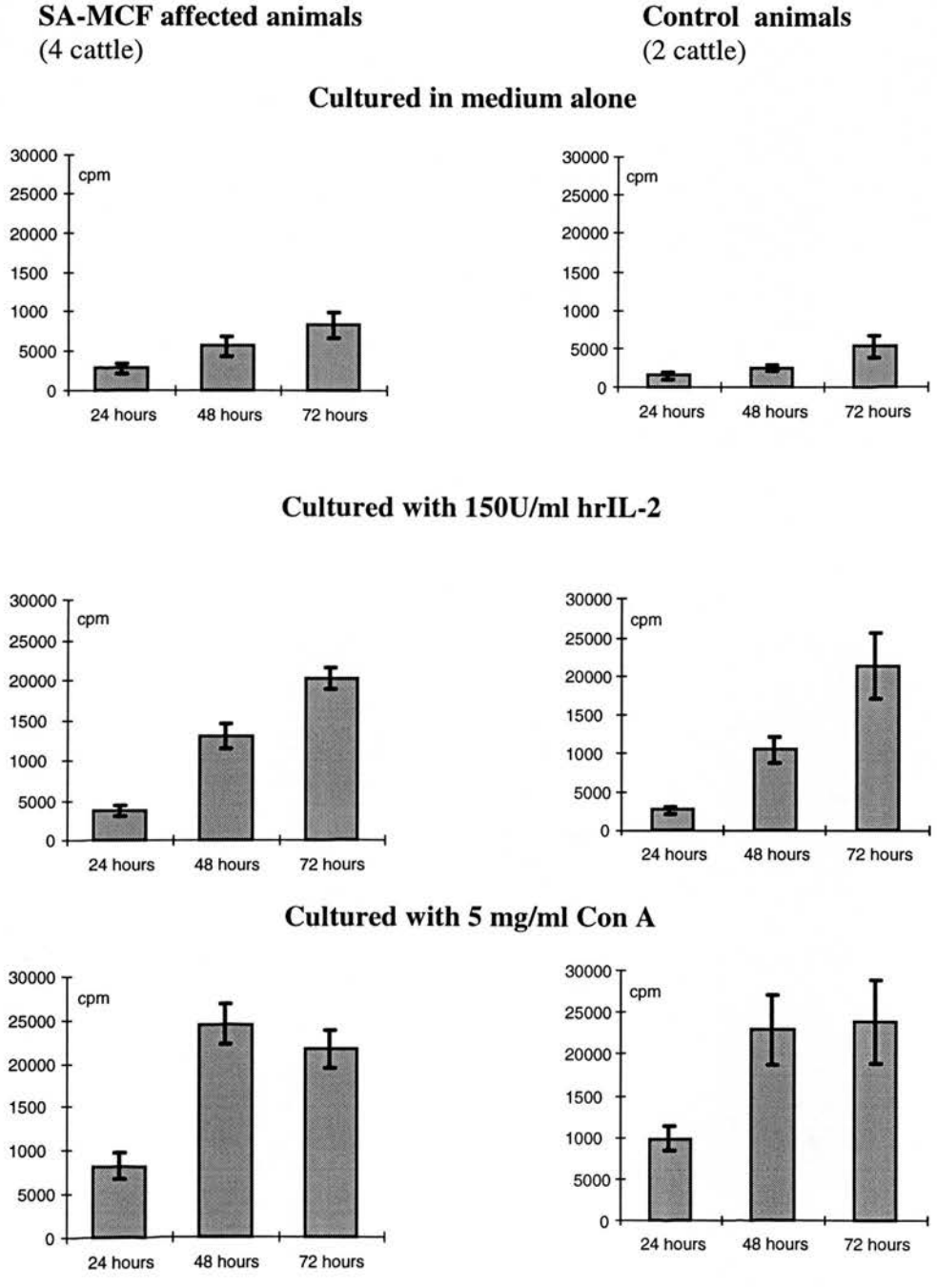
SI_{IL2} increased significantly ($p < 0.05$) over time from mean values of 1.4 (sem 0.07) at 24 hours and 4.9 (sem 1.8) at 48 hours, to 6.4 (sem 2.49) at 72 hours and was not significantly different from the results observed in short term cultures derived from AHV-1 infected rabbits (Chapter 5). No significant correlation was found between the grade of the hyperplasia and the response to exogenous IL-2.

Table 8. 3 Thymidine uptake (mean cpm ± sem) of short term cultures of mesenteric, prescapular, prefemoral and retropharyngeal lymph node cells derived from four SA-MCF affected cattle (BJ 971, BJ 1004, BJ 1044 and BJ 1187).

CC	Organ	24 hours	48 hours	72 hours
CM	M	2,547 ± 938	4,590 ± 1,833	6,471 ± 2,254
	PF	2,887 ± 1,495	7,004 ± 3,088	10,505 ± 3,424
	PS	2,529 ± 1,306	5,917 ± 2,991	9,455 ± 3,770
	RF	3,310 ± 1,936	4,812 ± 3,387	6,252 ± 4,506
IL-2	M	3,555 ± 1,226	12,524 ± 3,161	19,103 ± 3,902
	PF	4,297 ± 1,760	15,163 ± 2,653	22,401 ± 751
	PS	3,155 ± 1,410	12,565 ± 3,356	20,991 ± 1,239
	RF	4,141 ± 2,078	12,445 ± 4,220	18,247 ± 3,044
Con A	M	6,213 ± 2,030	25,308 ± 4,287	20,617 ± 3,789
	PF	7,336 ± 2,246	27,427 ± 2,982	24,691 ± 3,001
	PS	8,938 ± 3,191	25,329 ± 3,672	25,079 ± 2,878
	RF	11,090 ± 5,084	20,532 ± 9,840	14,276 ± 7,604

CC culture conditions; **CM** cultured with medium alone; **IL-2** cultures with 150 U/ml hrIL-2; **Con A** cultured with 5 mg/ml Con A; **M** mesenteric lymph node; **PF** prefemoral lymph node; **PS** prescapular lymph node; **RF** retropharyngeal lymph node

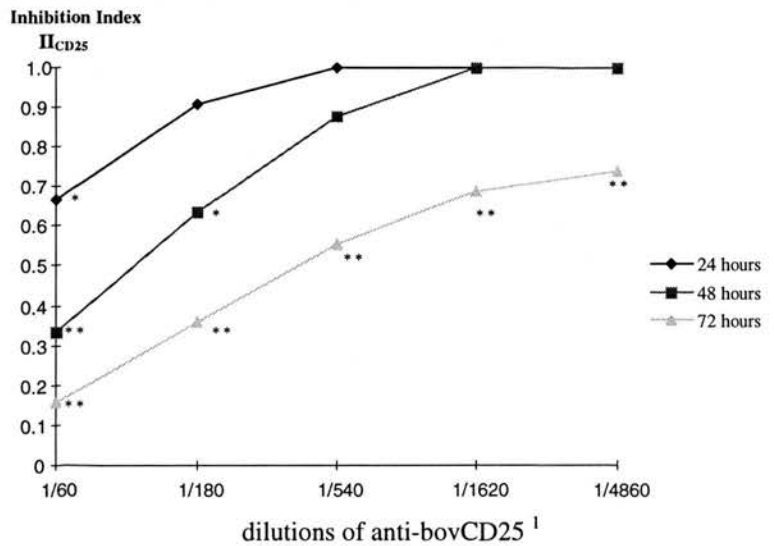
Figure 8. 4 Uptake of thymidine by short term cultures of lymph node cells derived from four SA-MCF affected cattle and two control animals and cultured with medium alone, IL-2 or Con A over a 72 hour period



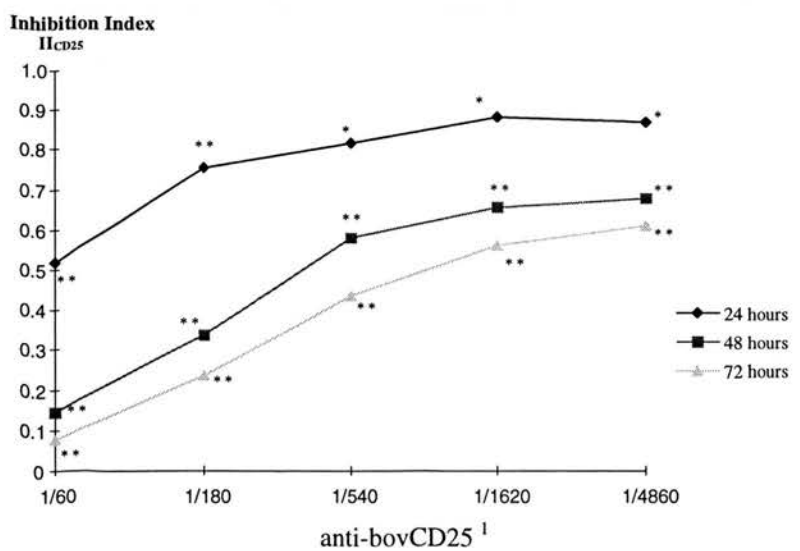
The growth of lymph node cells from case BJ 1004 was inhibited significantly ($p < 0.01$) by bovine anti-CD25 whereas the control antibody did not inhibit the growth of these cells (Figure 8.5 A). The cultures were significantly more inhibited when IL-2 was added and after longer incubation ($p < 0.01$) (Figure 8.5 B).

Figure 8. 5 Inhibition of the growth of freshly explanted retropharyngeal lymph node cells derived from a SA-MCF affected cow (BJ 1004) by anti-bovCD25 over 72 hours in culture

A- Lymphocytes cultured with anti bovCD25 and medium alone



B- Lymphocytes cultured with hrIL-2 and anti bovCD25



* $p < 0.05$
** $p < 0.01$
[1] dilutions of ascites

8.4 Discussion

8.4.1 Pathology of SA-MCF in cattle

Most of the cattle submitted to the Institute showed typical signs of the head and eye form of MCF (Table 8.2). Since, during experimental infections with SA-MCF, the intestinal form was observed at least as often as the head and eye form (Goetze and Liess, 1930; Pierson *et al.,* 1979), it must be assumed that typical gastro-intestinal and milder forms of MCF are generally not recognised as such and are frequently mis-diagnosed (Chapter 1.2.4). The diagnosis was based on histopathological changes and confirmed by serology. The PCR developed by Baxter et al., (1993) proved to be a valid method of diagnosis since the histopathological and serological data correspond well to the data obtained by this method of diagnosis. The PCR can, therefore, be used for ante-mortem diagnosis and for diagnosis of difficult cases such as cattle with the gastro-intestinal and mild form of MCF.

8.4.2 Short term cultures of bovine lymph node cells

The short term cultures of explanted lymph node cells derived from SA-MCF affected cattle showed that the multiplication of these cells increased over the period examined and that IL-2 as well as Con A increased the thymidine uptake. Thus no difference in behaviour of lymphocyte from MCF-affected animals and control animals was detected. The results are in strong contrast to the data obtained in the experimental studies of AHV-1 infected rabbits (Chapter 5). The only comparable feature between the two studies is the observed increase in thymidine uptake caused by exogenous IL-2. The difference could be the manifestation of a variety of factors:-

Cattle were affected by SA-MCF whereas rabbits were affected by WA- MCF

Even though no difference in the clinico-pathological lesions can be found between the lesions caused by the two viruses (Chapter 1.2.3), it is possible that the pathogenesis of the two forms of the disease is not identical.

One possible difference could be that the two viruses have different tissue tropism. Such a hypothesis is suggested by the observation that the different viruses cause

different pathology in the rabbit (Chapter 3) which might, therefore, explain the differences between short term lymph node cultures

Cattle were natural cases whereas rabbits were infected under controlled experimental conditions The cattle contracted the disease in their natural environment which probably involves cell-free OHV-2, whereas the rabbits were intravenously injected with a relatively high dose of cell-associated AHV-1. It can, therefore, be assumed that the initial establishment of the viral infection will be quantitatively different in cattle and rabbits.

Furthermore, the cattle were field cases and the animals were probably under stress from a number of factors including adverse weather, variability in diet, and other infections. The variability observed between the different cattle could be the expression of these circumstances. In contrast, rabbits were infected in controlled environments which excluded such variables.

The difference can be further explained by the fact that the incubation period was unknown in all cases. Since degenerative as well as hyperplastic changes are common in the lymph nodes of MCF-affected animals, it is possible that explanted lymphocytes behave differently depending on the length of the incubation period of the disease.

Furthermore, the control animals had different histories since one was suffering from mucosal disease while the other had been hyperimmunised. This means that the lymphocytes were potentially activated in both animals. Furthermore, foetal bovine serum present in the cell culture medium might have been mitogenic and the relatively high thymidine uptake in both groups, when cultured with medium alone, might be due to this effect.

The anatomy of the immune system of cattle and rabbit is different There are marked histological differences between cattle and rabbits such as the different staining affinities of neutrophilic granulocytes in cattle while the rabbit homologue is heterophilic (Kozma *et al.,* 1974). There are no comparative data concerning lymph node cell populations, but the proportions of blood cells differ between the two species (Table 8.4), leading to the assumption that lymph node cell populations differ

both anatomically and functionally. This might have a significant influence on the composition of the explanted lymph node cells, and even though prepared similarly, on the quantity and *in vitro* behaviour of virus harbouring cells.

Table 8. 4 Comparison of white blood cell (WBC) count between rabbit (New Zealand White) and domestic cattle

Parameter	Rabbit [1] (Kozma *et al.*, 1974)	Cattle [2] (Rosenberger, 1977)
WBC (x 10^3/mm^3)	8.03 - 13.28	7.5
Neutrophils or equivalent	6.38 - 19.88 %	33 %
Lymphocytes	73.00 - 90.88 %	55 %
Monocytes	0.73 - 3.25 %	5 %
Eosinophils	0.13 - 1.63 %	5 %
Basophils	1.13 - 3.63 %	0.5 %

[1] range for New Zealand White [2] mean value

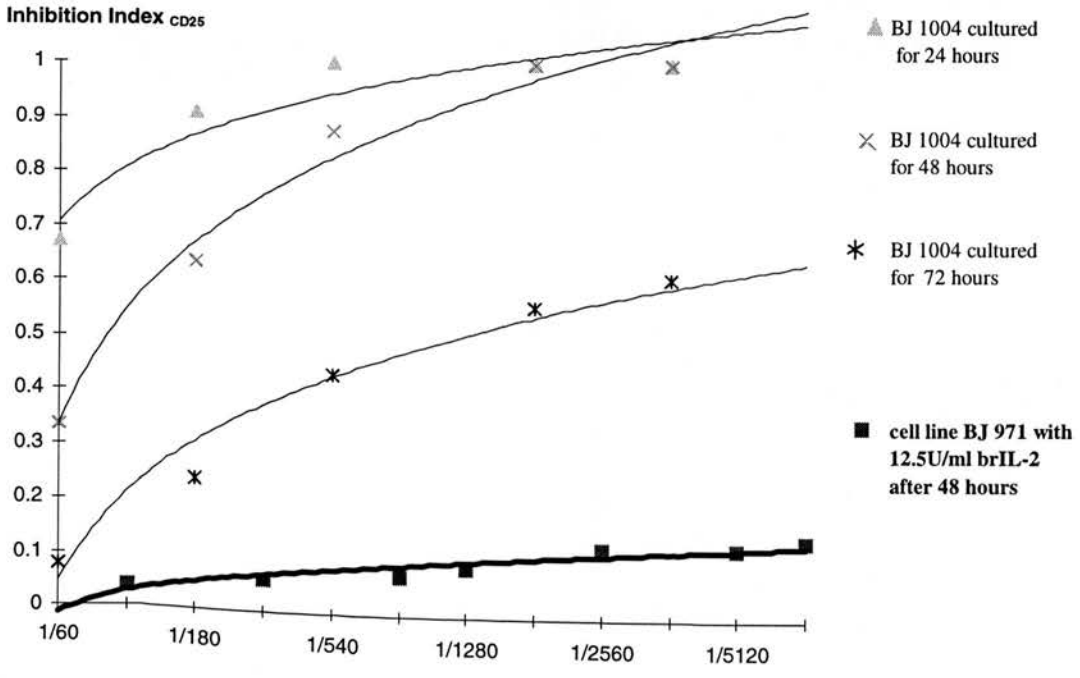
It could, therefore, be hypothesised that the data from the short term cultures reflect the effect of OHV-2 on the whole lymphocyte population *in vivo*, but that the effect of the virus infected cells on the whole organism is not detected when the bulk of the cell populations is examined in short term culture. For the initial understanding of the pathogenesis, it is, therefore, more appropriate to study the effect of OHV-2 in cell lines where viral DNA is present in a large proportion of such cells. The characterisation of OHV-2 harbouring cell lines will be considered in chapter 9.

8.4.3 Inhibition of the growth of bovine lymph node cells by bovine anti-CD25

Freshly explanted lymph node cells were cultured with anti-bovCD25 to determine whether the multiplication observed *in vivo* could arise through the effect of IL-2. It was expected that cell multiplication due to IL-2 produced by the cell population itself would be inhibited by this antibody. Cells were also cultured in the presence of IL-2 to evaluate the effect of the mAb more directly.

The culture of IL-2 unstimulated lymph node cells with anti-bovCD25 showed that cell growth could be inhibited significantly only after 72 hours. It should be noted that lymphocytes activated through IL-2Rβ and/or γ or through the IL-2Rα *in vivo* will not be influenced by the presence of anti-CD25 and that only those explanted cells expressing functional free IL-2Rα will be successfully inhibited by the presence of the mAb. This becomes apparent if the results obtained from freshly explanted lymph node cells are compared with the data obtained by *in vitro* growth of cell line BJ 971 (Chapter 9). The freshly explanted cells are only partially inhibited after 72 hours of incubation with the mAb whereas the cell line was fully inhibited after 48 hours (Figure 8.6). Since the thymidine uptake of untreated cells was relatively high it can be concluded that large numbers of cells were activated *in vivo* or through an IL-2Rα independent pathway. Furthermore, cell behaviour is altered as soon as cells are cultured *in vitro* and especially in this situation where it can be assumed that OHV-2 strongly influences the growth of the cells (Chapter 1.2.6.2). The validity of this experiment is questionable since too many variables have to be considered. It was therefore decided not to repeat the experiment on freshly explanted cells derived from other animals.

Figure 8. 6 Comparison of anti-CD25 neutralising activity on explanted retropharyngeal lymph node cells (BJ 1004) cultured in medium alone (results from this chapter) and on a IL-2 dependent bovine cell line (result presented in chapter 9)



8.4.4 Summary

It was possible to diagnose MCF by pathological, serological and molecular biological examination in 14 out of 15 cattle submitted to the MRI with suspected SA-MCF. Most of the cases showed clinical signs of the head and eye form of the disease. Even though the short term culture of freshly explanted lymph node cells of six animals showed no difference between MCF-affected and control animals, it was observed that:

1. thymidine uptake of cells derived from SA-MCF affected cattle cultured with medium alone increased over 72 hours and was correlated to the grade of hyperplasia
2. exogenous hrIL-2 increased the thymidine uptake and the increase was comparable to that observed in lymph node cells derived from AHV-1 infected rabbits
3. lymphocytes derived from SA-MCF affected cattle reacted to Con A in a comparable way to cells derived from control animals
4. anti-bovCD25 inhibited the cell growth increasingly over 72 hours in the one SA-MCF-affected calf studied

Chapter 9

Phenotype, growth regulation and cytokine expression of OHV-2 positive bovine cell lines

9.1 Introduction

Lymphocytes have been classified on the basis of their cell surface markers such as CD4 and CD8 as well as for their ability to secrete cytokines (chapter 1.2 and 4). These intercellular mediators are simple polypeptides or glycoproteins whose production is regulated mainly at the transcriptional level (reviewed by Vilcek and Le, 1991). Their effects are generally very localised and transient causing alteration in the pattern of gene expression in target cells which results in variation in the rate of cell proliferation and changes in their functional differentiation. The interplay between the secretion of cytokines and the expression of high affinity receptors on target cells creates a communication network between the different lymphoid and non lymphoid cells which directs the appropriate immune responses. The most thoroughly investigated cytokine is IL-2 which has already been described in detail (Chapter 1.2). Other cytokines such as IL-1, IL-4, IL-10, IFNγ and TNFα for which bovine sequence data were available are reviewed below and their major immunological activities are summarised in table 9.1.

The establishment of continuously growing T-cell lines from SA-MCF affected cattle, rabbits and deer has been described by various authors (Chapter 1.2.6.2). These cells have the characteristics of LAK cells (Chapter 1.2.6.2), being IL-2 dependent, capable of non MHC class II restricted cytotoxicity and expressing either CD4, CD8 or WC1 (bovine equivalent to γ/δ cells). In addition, it was shown by molecular biological methods that the cell lines contained at least part of the OHV-2 genome (Chapter 1. 1.2.2.2). Investigation of the functional properties of these cell lines is, therefore, important in order to establish an understanding of the behaviour of this virus in a homologous *in vitro* system which is likely to reflect the pathogenesis *in vivo*.

9.1.1 Interleukin-1 (IL-1 α and β)

IL-1 α and β are the product of two distinct genes, which recognise the same receptor and, therefore, share biological properties (reviewed by Dinarello, 1993, 1994; Saukkonenen *et al.,* 1990). There is some evidence that IL-1α may function as a

autocrine messenger in the skin and CNS, whereas IL-1β enters the circulation exerting a systemic effect. These cytokines are mainly produced by monocyte type cells, but also by endothelial cells, dendritic cells, lymphocytes (T, B and NK cells), keratinocytes, hepatocytes, fibroblasts, glial cells, thymic and salivary gland epithelium.

The cytokines are amongst the first detectable cytokines during an antigen driven immune response. Their functions are the activation of T- and B-cells, thereby inducing increased expression of IL-2R and of other cytokines such as IL-2 and IL-4. IL-1 is produced during traumatic, toxic and infectious processes as well as in neoplastic conditions such as monocytic leukaemia, Hodgkin's disease and adult T-cell leukaemia. IL-1 also plays a role in the pathogenesis of endotoxic shock and inflammatory diseases such as rheumatoid arthritis and graft-versus host disease. Its proinflammatory effect can be beneficial and is in fact used in vaccinology where IL-1 has been tested as an adjuvant.

It was shown that, in cattle, IL-1β is produced by monocytes after stimulation with antigen and that it modulates the response of B-cells to IL-2 (Lederer and Czuprynski, 1989; Kenefick *et al.,* 1992; Collins and Oldham, 1995). Furthermore, human recombinant IL-1β upregulated CD11a, CD18 and VLA-4 on bovine brain endothelial cells (de Vries *et al.,* 1994). Additionally, recombinant bovine IL-1β, induced significant accumulation of neutrophils and monocytes *in vivo* (Heidel *et al.,* 1990; Perrson *et al.,* 1993). It was also shown that recombinant IL-1β acts as a proinflammatory cytokine in cattle when used as an adjuvant in vaccines against BHV-1 (Reddy *et al.,* 1990). The administration of IL-1β increased the number of neutrophils and monocytes in peripheral blood and serum neutralising antibody titres were enhanced as were the cytotoxic responses against BHV-1 infected bovine kidney fibroblasts.

9.1.2 Interleukin-4 (IL-4)

IL-4 was initially discovered as a B cell stimulatory factor because of its ability to induce B-cell proliferation and differentiation such as the promotion of IgE and IgG1 switch in B-cells and the expression of activation markers CD23 and surface IgM

(reviewed by Banchereau and Rybak, 1994; Peace *et al.,* 1988). The effects of IL-4 on resting B-cells indicate that it enhances antigen-presentation to T-cells by B-cells. This T-B-cell interaction leads to the enhancement of antibody production. IL-4 is produced by Th_2 cells and mast cells, and there is conflicting evidence about the possibility that $CD8^+$ cells produce IL-4 (Kemeny *et al.,* 1994). Activated $CD4^+$ and $CD8^+$ cells proliferate in response to IL-4 in an IL-2 independent fashion, but the production of IFNγ, its natural antagonist, is blocked. Furthermore, IL-4 can induce expression of CD8 on $CD4^+$ T-cell clones and increase antigen specific cytotoxicity. IL-4 inhibits the production of cytokines by monocyte/macrophage as well as the killing of various intracellular parasites by these cells. Activated $CD3^-$ NK-cells proliferate in response to IL-4. It is also possible to induce LAK cell activity in mice and human lymphocytes by culturing in medium containing recombinant IL-4. On endothelial cells, IL-4 enhances the expression of VCAM-1 and increases the adhesiveness for T-cells, eosinophils, basophils and monocytes, but not neutrophils. It also activates fibroblasts inducing multiplication, collagen secretion and production of colony stimulating factors. Another interesting feature of IL-4 is its antitumour activity *in vivo.*

Recently it has been shown that, in cattle, IL-4 increases the expression of CD23, MHC class II, IL-2R and surface IgM in B-cells and enhances IgE and IgG1 production by these cells. Whereas B cells proliferate in response to recombinant bovine IL-4, T-cell proliferation was inhibited (Estes *et al.,* 1995)

9.1.3 Interleukin-10 (IL-10)

IL-10 is an inhibitory cytokine which represses proinflammatory cytokine expression such as IL-2, IFNγ and TNFα expression by macrophages, certain T-cells, granulocytes and NK-cells (reviewed by Mosman, 1994, Moore *et al.,* 1993). IL-10 is produced by murine Th_0 and Th_2 $CD4^+$ cell clones, but not by Th_1 or $CD8^+$ clones whereas human Th_2 and $CD8^+$ cell clones both produce this protein. IL-10 has an important role in regulating the equilibrium between the different T-cell subsets. It activates B-cells, like IL-4, favouring antibody responses and inhibiting delayed type hypersensitivity. However, in contrast to IL-4 which promotes IgG1, IgM and

IgE, IL-10 induces IgA, IgG1, IgG3 and IgD. Furthermore, IL-10 is produced by macrophages and B-cells after response to antigen or mitogens. In all cases, cells only produce IL-10 after antigen stimulation, indicating its role as a secondary mediator in the cytokine cascade. Keratinocytes also produce IL-10, particularly after exposure to ultraviolet light.

The most interesting aspect of this cytokine with regard to this study, is the fact that at least two other herpesviruses, namely EBV and EHV-2, carry homologues to this cytokine (Chapter 1.3). It is thought that the virokine may be advantageous to the virus since it inhibits the proinflammatory cytokine production and, therefore, could weaken the antiviral immune response while enhancing B-cell proliferation.

In cattle, it was shown that recombinant human IL-10 does not inhibit accessory cell dependent proliferation of Th clones specific for *Babesia bovis* and *Fasciola hepatica* by down regulation of IL-2 or IL-4, but rather by down regulation of IL-2Rα expression (Chitkomckown *et al.,* 1995). IFNγ production was also consistently down regulated in the presence of IL-10 in the same clones.

9.1.4 Interferon γ (IFNγ)

IFNγ was first described as an antiviral factor secreted by mitogen stimulated lymphocytes (reviewed by Maeyer and Maeyer-Giunard, 1992, 1994). Its production is restricted to antigen stimulated T-cells and NK-cells, in contrast to IFNα and β synthesis which can take place in most cells types. IFNγ is usually induced concurrently with IL-2 and up-regulates its own mRNA synthesis, but in contrast to IL-2 it has an anti-proliferative effect. Helper cells are the most important source of IFNγ and, in the murine system, it has been shown to be secreted by Th_1 clones and inhibits the proliferation of Th_2 clones. The tumouricidal and microbicidal activity of monocytes/macrophages as well as cytotoxic T-cells and NK-cells is enhanced by this factor due at least in part to the induction of other cytokines such as TNFα and IL-1 in macrophages. Furthermore, IFNγ upregulates MHC class I and class II antigens, TNF receptors and IL-2Rγ expression (receptor for IL-2, IL-4, IL-7, IL-9 and IL-15). Studies in gene knock-out mice lacking a functional IFNγ receptor have

shown that this cytokine is essential for the generation of a normal IgG2a and IgG3 response. In *in vivo* experiments (knock-out mice and antibody neutralisation studies), it was established that IFNγ is a critical mediator of endotoxic shock (Car *et al.,* 1994). Activation of the TCR in the absence of costimulatory signals from accessory cells leads to cell death via IFNγ production, suggesting that the cytokine is involved in a mechanism for eliminating autoreactive T cells.

In cattle, recombinant IFNγ enhances the expression of MHC class I and II antigens on bovine lymphocytes, reduces migration of bovine neutrophils and increases antibody mediated as well as antibody independent cytotoxicity (Steinbeck *et al.,* 1986; Walrand *et al.,* 1989). IFNγ is produced by peripheral blood cells upon stimulation with antigen which has been exploited to develop an antigen specific diagnostic test for *Mycobacterium bovis* infection (Wood *et al.,* 1992; Wood and Rothel, 1994). *In vivo* administrations of high doses of recombinant bovine IFNγ to healthy cattle induced fever (Roth and Frank, 1989). It is of special interest that only some bovine T-cell lines infected with *Theileria parva* and *T. annulata* produce IFNγ (Entrican *et al.,* 1991, Preston *et al.,* 1993, Ahmed *et al.,* 1993,).

9.1.5 Tumour necrosis factor α (TNFα)

TNFα, also known as cachectin, is a proinflammatory cytokine first described for its anti-tumour and anti-infection activity (reviewed by Piguet *et al.,* 1987; Jacob, 1992; Tracey, 1994). Like IL-1, it is mainly produced by cells of the monocyte/macrophage lineage, but also by granulocytes, keratinocytes astrocytes, T-, B- and NK-cells. Microbial products, most importantly LPS, IL-1, IL-2, GM-CSF and TNFα itself, trigger the secretion of TNFα whereas IL-10 and TGFβ inhibit its activity. TNFα induces the production of IL-1, IL-6, IL-8, IFNγ, GM-CSF and TGFβ. Like IL-1 and IFNγ, this cytokine is important in the pathogenesis of shock, having a multitude of effects on the cardio-vascular and neuro-hormonal system. By blocking TNFα activity with specific antibodies, it was shown that necrosis observed in autoimmune

disease such as lupus erythematosus and acute graft versus host disease could be attenuated.

It was shown that TNFα is released *in vitro* by bovine whole blood, peripheral blood monocytes and alveolar macrophages (Adams and Czuprynski, 1990, 1995; Bienhoff *et al.,* 1992). Treatment of bovine neutrophils with recombinant bovine TNFα (bovrTNFα) enhanced the antibody independent neutrophilic cytotoxicity, but did not affect *Staphylococcus aureus* ingestion or antibody dependent cell mediated cytotoxicity. Simultaneous treatment with bovrTNFα and bovrIFNγ had a synergistic effect (Chiang *et al.,* 1991). In addition, human recombinant TNFα and β induced apoptosis of bovine oligodendrocytes *in vitro* in a dose and time dependent fashion, whereas IL-2, IL-6 and IFNγ had no effect (Selmaj *et al.,* 1991).

Furthermore, it was shown that the administration of recombinant bovine TNFα to cattle induces a similar acute metabolic and hormonal response to that induced by bacterial endotoxins (Kenison *et al.,* 1991).

From this short review of the different cytokines, it can be summarised that, IL-1, IFNγ and TNFα are proinflammatory cytokines whereas IL-4 and IL-10 are mainly anti-inflammatory mediators. All cytokines have been implicated in inflammatory disease such as shock, autoimmune disease and graft versus host disease. Recently, the role of these cytokines in the central nervous system (CNS) has been investigated and it has been shown that most cytokines have local as well as systemic effects in the CNS, regulating the function of neurones and neuroglia as well as leukocyte trafficking in the CNS. These cytokines might, therefore, be involved in the mechanisms which induce the neuropathological lesions as well as the development of lesions in other tissues observed in MCF .

Table 9. 1 Principal immunological properties of the cytokines examined in this study

Effect / *Inducer*	IL-1	IL-2	IL-4	IL-10	IFNγ	TNFα	IL-2R	Ig	CT[1]	Pyrexia	shock	GvH	AI
IL-1	↑	↑[B]	↑		↑	↑				+	+	+	+
IFNγ	↑[B]	↑	↑	↓	↑	↑[B]	↑	IgG ↑	+[B]	+	+		+
TNFα	↑[B]	↑	↓	↓	↑	↑			+[B]	+	+[B]	+	+
IL-2		↑	↓		↑	↑	↑	IgG1 ↓	+[B]			+	+
IL-4	↓	↓				↓	↑[B]	IgE IgG1 ↑[B]	+[B]				+
IL-10		↓	↑		↓[B]	↓	↑[B]	IgA IgD IgG ↑					+

Ig type of Ig; **IL-2R** induction of IL-2R; **CT** cytotoxic activity by T-cells, NK-cells and macrophage type cells; **GvH** graft versus host reaction; **AI** autoimmune disease and mechanisms of self-tolerance regulation; ↑ synergistic effect; ↓ negative effect; + effect. Data relate to bovine cytokines (indicated with [B]) as far as possible, but the other data derive from human and mouse models for reference see text; [1] also Jansen and Schultz, 1990

9.1.6 Transcriptional control of cytokines and cytokine receptor

Cytokines are inducible proteins and their production is mainly controlled at the level of transcription. At pre-transcriptional level, the specific cytokine gene is activated by stimulation of specific receptors, mainly the TCR complex but also other co-stimulatory receptors, which induce distinct intracellular signal transduction pathways. At post-transcriptional level, the accumulation of cytokine mRNA is inhibited due to the lability of the transcripts (AUUUA repeats).

The effect of cytokines is regulated in a comparable way, through stimulation of the cytokine receptor inducing effector genes, such as genes for functional proteins and genes involved in cell multiplication.

Through stimulation of the specific receptors a cascade of kinases is activated. The resulting phosphorylation of the pathway-specific factors allows the binding of these to promoter regions in the gene which is to be induced. Different pathways are known depending on the respective transcriptional factors. The best investigated transduction pathways for cytokine induction and their effects through stimulation of the specific receptor are those regulated either by the transcription factor STAT, NF-κB or NF-AT (as reviewed by Mustelin and Altman, 1993; Hill and Treisman, 1995; Ihle, 1995).

Cyclosporin A (Cs A) interferes with the NF-ATp pathway inhibiting calcineurin. The administration of Cs A has, therefore, a negative effect on the transcription of IL-2, IL-3, IL-4, GM-CSF, TNFα, IFNγ and probably other cytokines. It is, therefore, an ideal candidate to establish if cell growth is dependent on autogenous cytokine production (reviewed by Rao, 1994).

FK506 (a pharmacological derivative of Cs A), pentoxifyllin, rapamycin or other inhibitors which interfere with the regulation of a more restricted range of cytokines at either pre- or post-transcriptional level were considered, but were not available for this study.

9.2 Materials and Methods

9.2.1 Establishment of OHV-2 positive cell lines

Cells derived from lymph node, cerebral spinal fluid (CSF) and cornea of the cattle described in the previous chapter were cultured at a concentration of 2 x 10^6 cells/ml in 10 ml flasks with culture medium alone, 150 U/ml IL-2 and 5 µg/ml Con A to establish cell lines. The respective media were partially changed every three to four days and the cultures split if necessary. Strongly adherent cells were passaged by trypsinisation whereas slightly adherent cells were detached with the help of glass beads.

The presence of OHV-2 specific DNA was determined by PCR (Chapter 8.2.2).

9.2.2 Antibodies

The antibodies shown in table 9.2 were used for the phenotypic characterisation as well as for specific cell growth inhibition. The mAb CC20, CC42, CC30, CC8, CC17, CC63 and CC15 were kindly provided by Dr. C. Howard (Institute for Animals Health, Compton, UK). The mAb IL-A 111 and IL-A 109 were kindly provided by Dr. D. MacKeever (ILRI, Kenya). The polyclonal antibody raised against bovine IFNγ was kindly provided by Dr. G. Entrican (MRI, Edinburgh, UK). As control antibodies, a cocktail of five mAb of different isotypes against Border disease virus (BDV) was kindly provided by D. Dean (MRI, UK).

Table 9. 2 Antibodies used for the characterisation of OHV-2 positive cell lines

mAb	specificity	reference
CC20	CD 1	Parsons and MacHugh, 1991
CC42	CD 2	Davis and Splitter, 1991
CC30	CD 4	Bensaid and Hadam, 1991
CC8	CD 4	Bensaid and Hadam, 1991
CC17	CD 5	Howard and Leibold, 1991
CC63	CD 8	MacHugh and Sopp, 1991
IL-A 111	CD 25	Naessens *et al.*, 1992
CC15	WC1	Morrison and Davis, 1991
IL-A 109	Monocyte	Hall *et al.*, 1993

9.2.3 Phenotypic characterisation of OHV-2 positive cell lines

Non adherent cells were characterised by FACScan (Chapter 2.3.2) with anti bovine CD1, CD2, CD4, CD8, WC1 and CD25 (Table 9.2) whereas the adherent cell line BJ 971 was examined by staining the cell with mAb IL-A 109 by indirect immunoperoxidase (Chapter 2.3.1). The technique was slightly adapted since cells had to be grown on chamber slides for at least 24 hours and were only fixed with 1% paraformaldehyde after the staining with the primary antibody to ensure comparability with FACS analysis.

9.2.4 Characterisation of IL-2 independent adherent cell lines BJ 971 and BJ 1004

IL-2 independent adherent cell lines derived from cases BJ 971 and BJ1004 were characterised for the presence of α–naphthyl-acetate-esterase (αNAE). Cells were grown on glass slides, fixed with 4% paraformaldehyde for 10 min and stained for

the presence of αNAE using the α-Napthyl acetate esterase kit (Cat. No. 61A; Sigma, UK) following the manufacturer's instructions.

To investigate the phagocytic activity of these cells, autoclaved graphite was added generously to the cell culture tube. After four hours the monolayer was washed twice with PBS (Chapter 2.6) and the location of the graphite examined under the microscope.

9.2.5 Cell growth analysis

Washed cells from the various IL-2 dependent cell lines were harvested three days after the last IL-2 stimulation to be able to compare IL-2 stimulated and unstimulated cells. Cell growth was examined by incorporation of thymidine (Chapter 2.4.7). The cells were incubated at a concentration of 1 x 10^5 cells/well for variable intervals of time with medium supplemented with brIL-2, anti bovine CD 25, Concanavalin A (Con A), Cyclosporin A (Cs A) or anti bovine IFNγ.

The efficiency of rhIL-2 and brIL-2 at enhancing cell proliferation was evaluated by incubating the cells for 48 hours with doubling dilutions of the respective IL-2 starting at 50 U/ml. The specificity of the effect of IL-2 was established by incubating cells for the same time with and without IL-2 stimulation (25 U/ml hrIL-2 or 12.5 U/ml) for 48 hours and blocking the effect with various concentrations of anti-bovine CD 25 (Table 9.2).

The effect of Con A was established by incubating stimulated (150 U/ml rhIL-2) and unstimulated cells for 48 hours with 40, 20, 10, 5, 2.5 and 1.25 µg/ml Con A (Chapter 2.4.5). The effect of Con A was expressed by the stimulation index ($SI_{Con\ A}$):

$$SI_{Con\ A} = \frac{cpm_{IL2}}{cpm_{medium}}$$

The inhibitory effect of Cs A was evaluated by incubating the cells for 24, 48 and 72 hours without IL-2 or with 37.5 U/ml, 75 U/ml and 150 U/ml IL-2. Each dilution of IL-2 was further supplemented with 50, 100, 200 or 400 ng/ml Cs A (Chapter 2.4.5). The inhibition by Cs A was expressed for each concentration of IL-2 and Cs A by the inhibition index $II_{Cs\ A}$:

$$II_{Cs\ A} = \frac{cpm_{control}}{cpm_{CsA}}$$

This index expresses the degree of inhibition obtained by the single dilution of Cs A with respect to the specific control.

Attempts were made to influence cell growth by incubating the IL-2-stimulated and control cells from cell line BJ 1004 for 24 and 52 hours with polyclonal anti-IFNγ at dilutions of 1/200 to 1/200,000 (Entrican *et al.,* 1991).

9.2.6 Cytokine profile determined by RT/PCR Southern blot and restriction enzyme analysis

RNA was prepared from IL-2 stimulated and unstimulated cell lines BJ 971, BJ 1004, BJ 1035, BJ 1044 and Con A blasts derived from a healthy cow (Chapter 2.5.1). The RNA was transcribed to cDNA with random primers and diluted 1:5 in water (Chapter 2.5.4). The cDNA (5 µl/reaction) was amplified with two primer sets for IL-1β, three primer sets for IL-2, two primer sets for IL-4, one primer set for IL-10, two primer sets for IFNγ, two primer sets for TNFα and one primer set for β actin (Chapter 2.5.6). The specificity of the PCR product was confirmed by Southern blot or restriction enzyme analysis (Table 9.3) (Chapter 2.5.8). Digestion with the specific enzymes was performed following the manufacturer's instructions and the products examined on sodium dodecyl sulphate - polyacrylamide gels (SDS-PAG) and the size determined by comparison with a 1 kbp marker (Boehringer Mannheim, UK). Samples were run on a 7.5% polyacrylamide discontinuous gel with 3% stacking gel at 200V for the appropriate time and DNA was visualised by silver nitrate as described by Herring *et al.,* (1982).

Table 9. 3 Oligonucleotides and restriction enzymes used for the detection of cytokine mRNA

Cytokine	Oligonucleotide	Product	RE
IL-1β[1]	tga cgc acc cgt tca gtc aat * agt gaa gtt cag gct gca gct	564: 317 / 247	Pvu II
	gaa gtg atg gct tac tac ag *** aga ggt ggc cag gat ata ac	742	-
	cga tga gct tct gtg tga tgc agc cgt gca gtc agt aaa a *** tat tcc tct tgg ggt aga ctt tgg ggt cta ctt cct cca g	p	-
IL-2[2]	tgc tgc tgg att tac agt tgc * gag gca ctt agt gat caa gct	373: 224 / 149	Xbal
	atg tac aag ata caa ctc *** cat cta ctc aac aat gac	463	-
	att gaa aca tct tca gtg tct a ** cca ttt gtt cag aaa ttc tac a	206	-
	aga aga act caa acc tct gga gga agt gct taa ctt agc t **	p	-
IL-4[3]	gtc tca cct acc agc tga tc * tca gcg tac ttg tgc tcg tc	348: 207 / 141	Pst I
	atg ggt ctc acc tcc cag *** gag aaa tac tca aag tgt tga	407	-
	caa gag gtc tct cag cgt act tgt act cgt ctt ggc ttc a *** gac tgg aat tga gct tag gcg tat cta cag gag cca cat g	p	-

continued →

Table 9.3 continued

Cytokine	Oligonucleotide	Product	RE
IL-10[4]	aca gct cag cac tgc tct gtt * cgt tgt cat gta gga ttc tat g	518: 295 / 223	BstE II
IFNγ[5]	gca agt agc cca gat gta gc * ggt gac agg tca ttc atc ac	316: 164 / 152	EcoR V
	cca taa cac agg agc tac cg *** tct cag ggt cca act tgg ca	789	-
	gaa gtc ctc cag ttt ctc aga gct gcc gtt caa gaa ctt c *** ctg tgt gct ttt ggg ttt ttc tgg ttc tta tgg cca ggg c	p	-
TNFα[6]	act cag gtc atc ttc tca agc c * atg atc cca aag tag acc tgc c	464: 355 / 109	Bgl I
	ttg cag gag cca cca cgc tc *** tca gtg ctg aga tca acc tg	520	-
	cct caa ata aca agc cgg tag ccc acg ttg tag cca aca t*** ttg atg gca gag agg atg ttg acc ttg gtc tgg tag gag a	p	-
β actin	cca gac agc act gtg ttg gc * gag aag ctg tgc tac gtc gc	280	-

product size of PCR products and its digests in bp; **RE** restriction enzymes (all purchased from Boehringer Mannheim, UK); **p** internal probe; **-** not applicable; * designed and kindly provided by Dr. R.A Collins; **designed on basis of the rabbit sequence *** designed by Dr. C. McInnes

[1] Leong *et al.,* 1988; [2] Reeves *et al.,* 1987; [3] Heussler *et al.,* 1992; [4] Hash *et al.,* 1994; [5] Ceretti *et al.,* 1986; [6] Bienhoff and Allen, 1995

9.3 Results

9.3.1 Establishment of cell lines

Cell lines established from MCF-affected animals were either IL-2 dependent or independent and two types of morphologies were observed. Independently of their type, all the established cell lines were PCR positive for OHV-2 DNA.

IL-2 dependent cell lines which grew for at least two months were established from lymph nodes and CSF of eight cattle (Table 8.3). The cells grew rapidly and had to be expanded every three to four days. The cells were slightly adherent, initially pleiomorphic and formed aggregates. Cornea explants also grew well under these conditions. Initially, these cultures consisted of adherent fibro-epithelial cells. After two to three months in culture, these cells detached and appeared morphologically similar to those of lymph node and CSF lines. No growth was obtained with any of the cell lines at low cell density and when total medium was exchanged completely. Cryopreservation was not possible, because, after resuscitation, the cells would grow only for a short period.

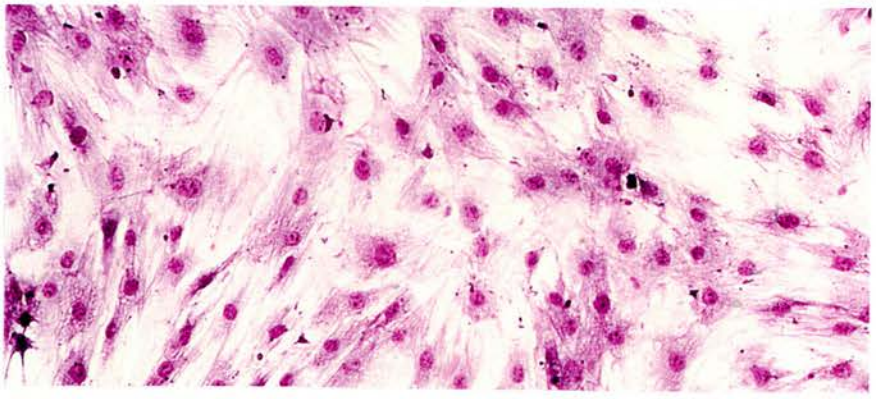
Attempts were made to establish cell lines by stimulating lymph node cells with Con A at intervals of 3 days. After initial multiplication for four to six days, the cells started to die and could only be rescued by exogenous IL-2. The rescue led to the establishment of IL-2 dependent cell lines with the same morphology and growth characteristics as cells lines derived without the use of Con A.

Two cell lines derived from animals BJ 971 and BJ 1004 were established from lymph nodes without IL-2 stimulation. The morphology was significantly different from the IL-2 dependent cell lines and was characterised by strongly adherent cells with the morphology of fibroblasts or macrophage type cells (Figure 9.1a). The cells grew as monolayers, were not inhibited by contact, phagocytosed graphite, stained with the antibody IL-A 109 (Figure 9.1b) and were αNAE positive, an esterase which is specific for macrophage-type cells (Thompson, 1966) (Figure 9.1c). Since these cells were derived from single cell suspension which were gradient purified, it is probable that these cells are monocyte type cells When supplemented with exogenous

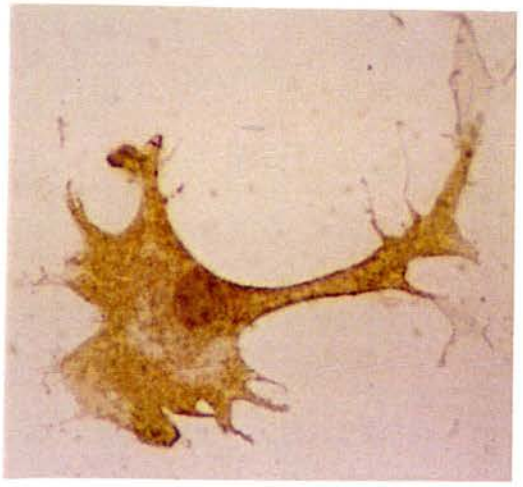
IL-2 the cultures gave rise to IL-2 dependent cell lines in a similar pattern to that observed in the cornea cell cultures. The cells started to detach after a number of passages (7-10) and became morphologically similar to the IL-2 dependent cell lines.

Figure 9. 1 Characterisation of the IL-2 independent OHV-2+ cell line BJ 971

A- Characteristic monolayer stained with Leishman



B- Cell labelled with mAb IL-A 109



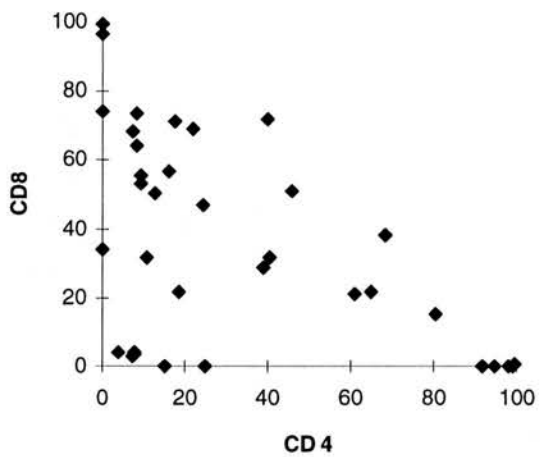
C- Cell stained for αNAE

9.3.2 Phenotypic analysis

The phenotypic analysis of eleven cell lines with mAbs to CD2, CD4, CD8 and CD5 showed that two cell lines (BJ 889 and BJ 913) lost their cell surface markers completely whereas all the other cell lines (BJ 1004, BJ 1035, BJ 1044, BJ 971, BJ 1104) were CD5 and CD2 positive (Table 9.4) and CD1 negative (**CD5**: mean = 89.35 % sem 3.0 sd 14.7; **CD2** mean = 86.6 % sem 2.1 sd 13.2)

Most of the cell lines were pure $CD4^+$ or $CD8^+$ T-cells, but frequently even after long periods in culture the populations were mixed (Table 9.4 and Figure 9.3 and 9.4). In the mixed population, there was a negative correlation between CD4 and CD8 positive cells (Figure 9.2). Cell lines BJ 971, BJ 1104, BJ 1044, BJ 1035 and BJ 1004 were 74.16 ± 3.8 % CD25 (Figure 9.4)

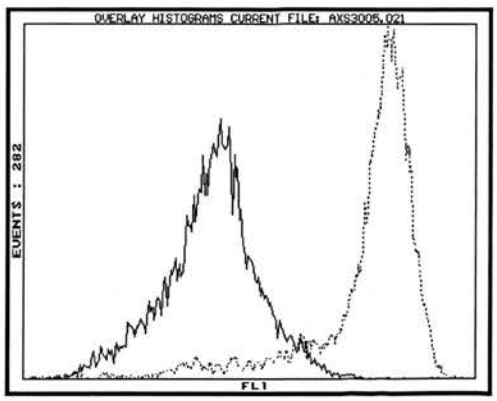
Figure 9. 2 Correlation* between CD4 and CD8 positive cells in OHV-2+ cell lines



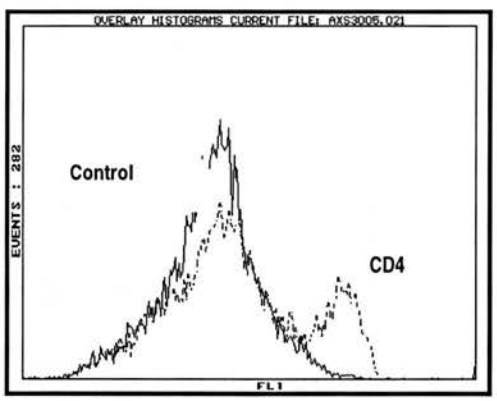
* Spearman's rank correlation coefficient R = -0.51 ($p < 0.01$)

Figure 9. 3 Phenotypic analysis of IL-2 dependent OHV-2^{+} cell line BJ 1044 by FACScan

A- Labelling with anti-CD2 (mAb CC42)



B- Labelling with anti-CD4 (mAb CC8)



C- Labelling with anti-CD8 (mAb CC63)

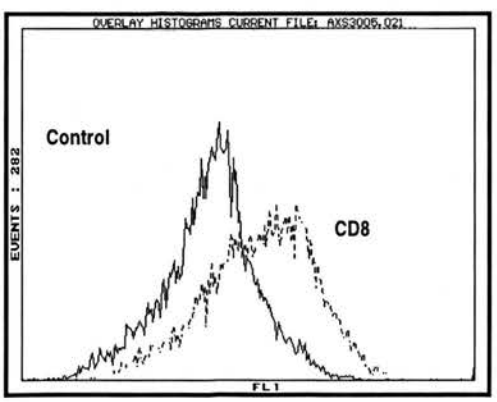
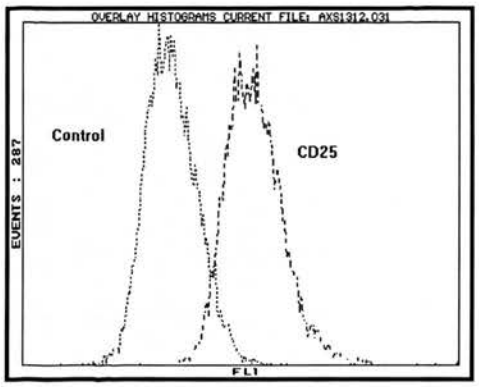
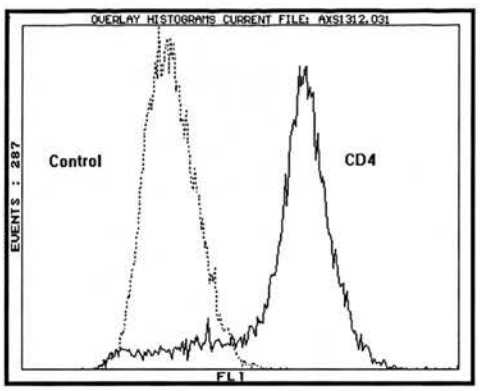


Figure 9. 4 Labelling of OHV-2^{+} cell line BJ 1004: this cell line is IL-2 dependent and the FACScan was performed 48 hours after stimulation with hrIL-2

A - Labelling with anti-CD25 (mAb IL-A III)



B - Labelling with anti-CD4 (mAb CC8)



C - Labelling with anti-CD2 (mAb CC42)

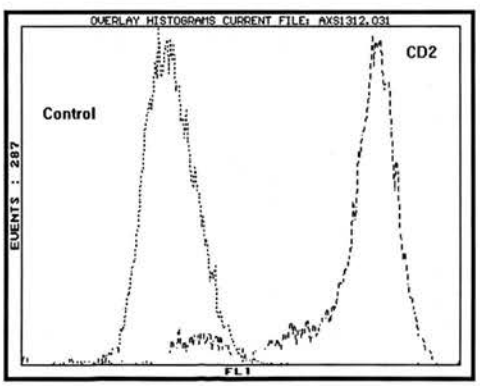


Table 9. 4 Phenotypic analysis of OHV-2 positive cell lines (percentage of positive cells)

Cell line	Tissue	IL-2	Time in culture	CD 5	CD 2	CD 4	CD 8	γ/δ
BJ 971	CSF	na	0 m			**46.0**	**51.0**	16.0
	LGL	+	6 m		97.58	**0.0**	**94.41**	
	LGL	+	7 m	96.02	95.04	3.1	**96.56**	
	LGL	**+**	***8 m***	***96.0***	***94.0***	***2.5***	***96.6***	***0.0***
BJ 1004	Cornea	+	16 m	84.6	86.8	**24.9**	0.0	0.0
	Cornea	+	24 m	84.0	69.6	**24.9**	0.0	0.0
	LGL	+	3 m	61.8	56.5	9.1	**55.5**	0.0
	LGL	**+**	***21 m***	***94.8***	***97.48***	***91.8***	***0.0***	
	LGL	+	22 m	95.0	97.5			
	LGL	+	26 m	56.5	64.6	9.1	**53.5**	0.0
	M/7	+	2 m	96.2	98.2	3.7	4.1	**46.3**
	M/7	+	3 m	96.7	97.6	8.0	3.8	0.0
	M/7	n	3 m	96.2	98.2	7.4	2.9	0.0
	M/7	+	21 m	98.1	97.8	**98.0**	0.0	0.0
	RF/4	+	3 m	96.7	97.6	8.0	3.8	**43.4**
	RF/4	+	21 m	97.0	97.7	**15.0**	0.0	
	RF/4	+	22 m	85.0	76.9			
BJ 1035	LGL	+	6 m	98.0	95.0	0.0	**34.0**	
	LGL	+	10 m	98.7	98.7		0.0	
	LGL	**+**	*11 m*	*99.4*	*99.8*	***99.5***	*0.3*	
BJ 1044	LGL	+	5 m	99.0	97.8			
	LGL	+	9 m	99.5	99.4	**68.2**	**37.9**	**33.5**
	LGL	**+**	***10 m***	***99.5***	***96.3***	***39.8***	***72.0***	
BJ 1104	LGL	+	1 m	92.3	93.5	0.0	**74.1**	
	LGL	**+**	***6 m***	***99.9***	***99.8***	***98.9***	***0.0***	

continued →

Table 9.4 continued

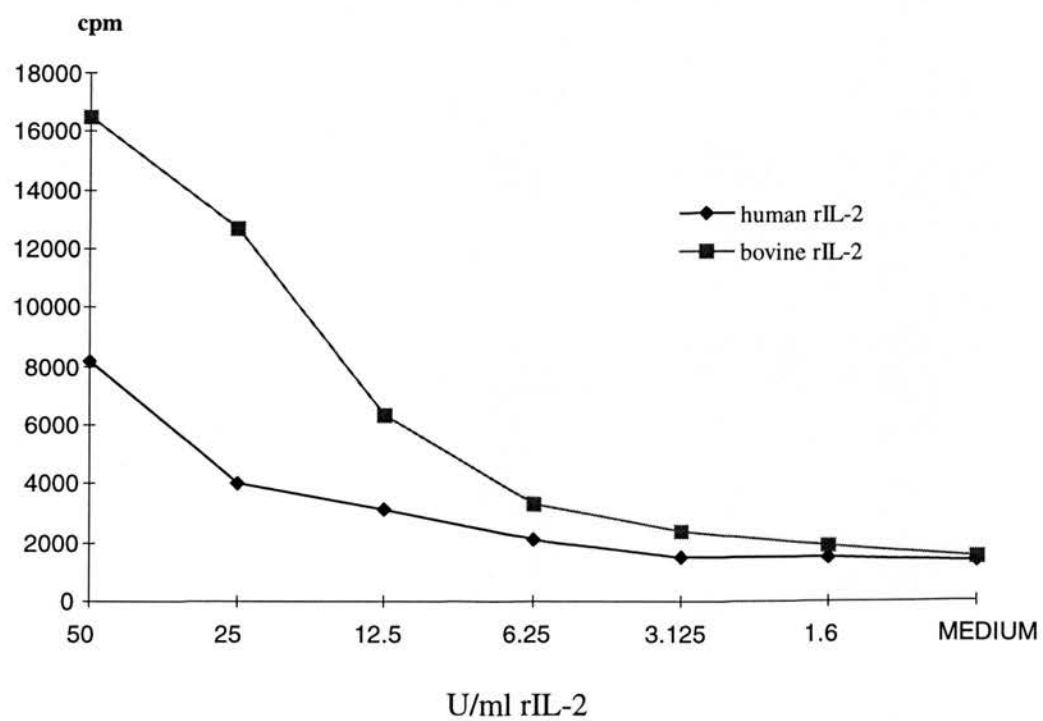
Cell line	Tissue	IL-2	Time in culture	CD 5	CD 2	CD 4	CD 8	γ/δ
BJ 884	CSF	+	3 m		75.2	8.3	**73.6**	15.7
	CSF	na	0 m			**18.4**	**21.9**	**19.8**
BJ 889	CSF	+	1 m		99.2	0.0	**99.4**	0.0
	CSF	+	2 m		0.0	0.0	0.0	0.0
	CSF	+	5 m		0.0	0.0	0.0	0.0
	CSF	+	6 m		0.0	0.0	0.0	0.0
	CSF	+	7 m		0.0	0.0	0.0	0.0
BJ 913	CSF	+	3 m		0.0	0.0	0.0	0.0
	CSF	+	4 m		0.0	0.0	0.0	0.0
	LGL	+	4 m		0.0	0.0	0.0	0.0
	LGL	+	5 m		0.0	0.0	0.0	0.0
BJ 924	CSF	n	1 m		91.9	**94.4**	0.0	0.0
	CSF	+	1 m		94.9	**80.4**	**15.0**	0.0
	CSF	+	2 m		89.9	**64.9**	**21.5**	0.0
	CSF	+	3 m		85.5	**38.8**	**28.9**	0.0
	LGL	+	1 m		73.7	8.2	**64.4**	0.0
	LGL	+	2 m		78.5	**60.8**	**21.1**	0.0
	LGL	+	3 m		79.0	**16.3**	**56.6**	0.0
BJ 934	CSF	+	1 m	49.6	75.1	7.4	**68.4**	0.0
	LGL	n	1 m		80.0	**24.3**	**46.6**	0.0
	LGL	+	1 m		83.7	**40.7**	**31.3**	0.0
	LGL	+	2 m		90.8	**22.2**	**69.2**	
	LGL	+	2 m		76.9	**17.5**	**71.6**	2.7
	LGL	n	2 m		48.7	**10.6**	**31.5**	0.0
	LGL	+	2 m		74.6	**12.8**	**50.2**	5.0

na not applicable, **n** cultured without IL-2, **LGL** large granular lymphocytes derived from lymph nodes, **CSF** cells derived from CSF; **M/7** initially adherent cells derived from mesenteric lymph node supplemented with IL-2 from passage 7; **RF/4** initially adherent cells derived from retropharyngeal lymph node supplemented with IL-2 from passage 4; *cell lines* used for further experiments are shown in italic

9.3.3 Effect of IL-2 and anti-CD25 on the growth of OHV-2 positive cell lines

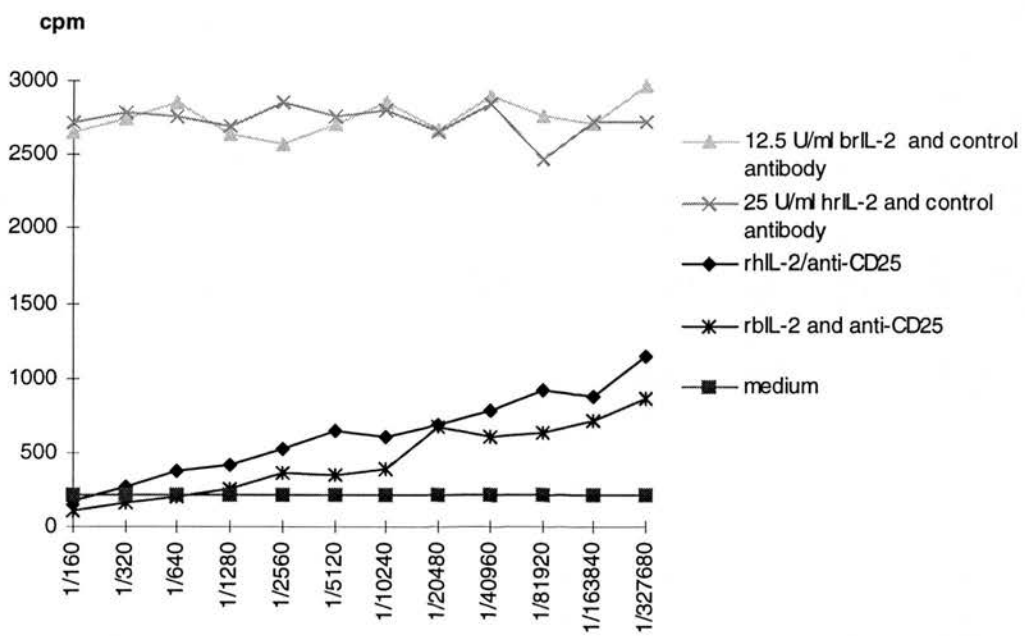
IL-2 dependent cell lines BJ 971, BJ 1004, BJ 1035, BJ 1104 and BJ 1044 responded to bovine and human recombinant IL-2 in a dose dependent manner, but bovine IL-2 seemed to have a stronger effect than human recombinant IL-2 (Figure 9.5).

Figure 9. 5 Uptake of thymidine by cell line BJ 1004 stimulated for 48 hours with human or bovine recombinant IL-2



IL-2 induced proliferation of cell lines BJ 971 and BJ 1004 could be blocked by bovine anti-CD25 (IL-A III) in a dose dependent manner, whereas the control antibody did not have any effect on the thymidine uptake (Figure 9.6). The mAb IL-A III had a very high titre and the IL-2 base line was never achieved even at a dilution of 1/327,680.

Figure 9. 6 Thymidine uptake of cell line BJ 971 cultured with human or bovine IL-2. Effect of dilutions of anti-bovine CD25 (IL-A III) after 48 hours incubation



IL-A III

9.3.4 Effect of Con A on OHV-2 positive cell lines

The reaction to Con A of the seven cell lines tested was significantly ($p < 0.01$) different from Con A blasts derived from lymph nodes of control animals (Table 9.5). The dose of 5 µg/ml Con A had only a slight effect on four cell lines (BJ 1187, BJ 1035, BJ 1044 and BJ 1196), whereas three cell lines (BJ 971, BJ 1004 and BJ 1104) did not respond to it (Figure 9.7).

Table 9. 5 Response of OHV-2 positive cell lines to Con A (5 µg/ml)

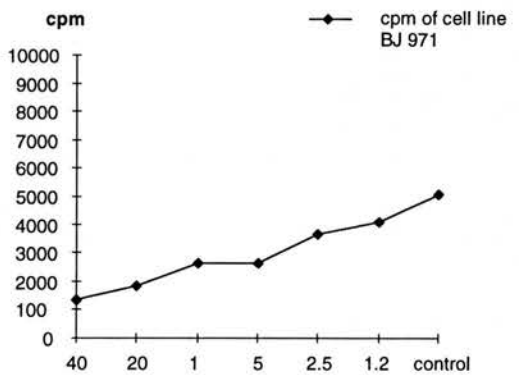
Cell lines	**SI** $_{\text{Con A}}$	**cpm** $_{\text{medium}}$
BJ 1187	10.1	1685
BJ 1035	5.5	733
BJ 1044	3.8	136
BJ 1196	2.1	2598
BJ 1004	1.2	5045
BJ 971	0.5	213
BJ 1104	0.5	60
Control[1]	12.3[1] ± sem 2.32	2521[2] ± sem 344

[1]mean value ± Con A blasts derived from seven control animals

[2] mean value ± of the same Con A blasts

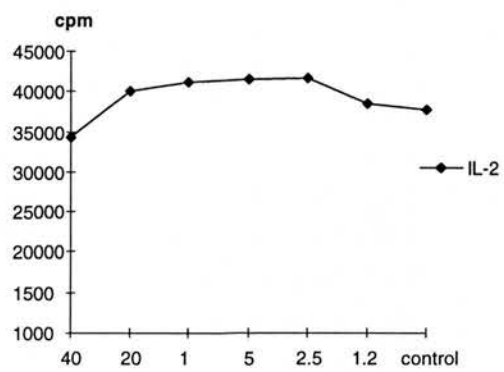
Figure 9. 7 Thymidine uptake by OHV-2 positive cell line BJ 971 cultured with or without IL-2 and treated with various concentrations of *Con A*

A- cultured with medium alone



mg/ml Con A

B- cultured with 150 U/ml hrIL-2



mg/ml Con A

thymidine uptake of cell line BJ 971

9.3.5 Effect of Cs A on OHV-2 positive cell lines

Cs A inhibited significantly ($p < 0.001$) the growth of the IL-2 stimulated and medium control cell lines (Table 9.6)

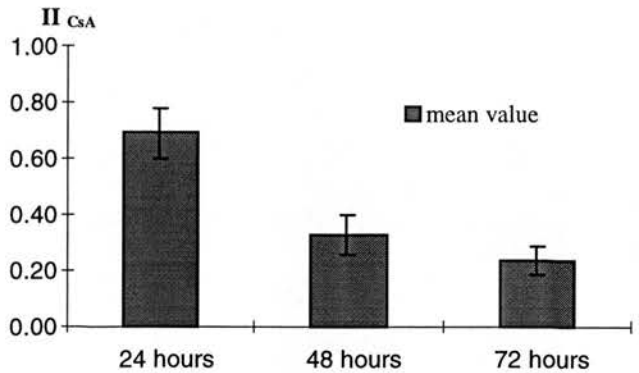
Table 9. 6 Effect of Cs A on IL-2 stimulated and unstimulated OHV-2 positive cell lines at 24, 48 and 72 hours

IL-2	Time	Cs A				
	(hours)	Control	50 ng/ml	100 ng/ml	200 ng/ml	400 ng/ml
medium	24	844[1] ± 244[2]	619 ± 197	603 ± 196	546 ± 166	541± 161
	48	2098 ± 931	710 ± 414	325 ± 919	199.5 ± 37	171.3 ± 33
	72	1830 ± 906	553 ± 384	240 ± 106	174.5 ± 78	80.8 ± 18
37.5 U/ml	24	3431 ± 1174	2976 ± 1027	2786 ± 1000	2627 ± 986	2275 ± 891
	48	9883 ± 2254	6021 ± 591	5588 ± 367	5356 ± 526	4692 ± 564
	72	11880 ± 1798	5930 ± 2489	5542 ± 2108	4337 ± 1396	3952 ± 1228
75 U/ml	24	3851 ± 1506	3403 ± 1249	3149 ± 1159	3026 ± 1137	2946 ± 1134
	48	11938 ± 1912	9945 ± 749	9737 ± 906	9632 ± 534	8405 ± 688
	72	15505 ± 2235	8552 ± 1832	8855 ± 2190	7240 ± 1239	6743 ± 1937
150 U/ml	24	3562 ± 1678	3364 ± 1568	3147 ± 1410	3062 ± 1313	3087 ± 1364
	48	13305 ± 611	12744 ± 551	12968 ± 506	11481 ± 708	11123 ± 319
	72	18486 ± 2001	14717 ± 1653	13095 ± 1756	10451 ± 2210	10275 ± 1946

[1] mean value of cell lines BJ 1004, BJ 1035, BJ 1044 and BJ 1104; [2] sem

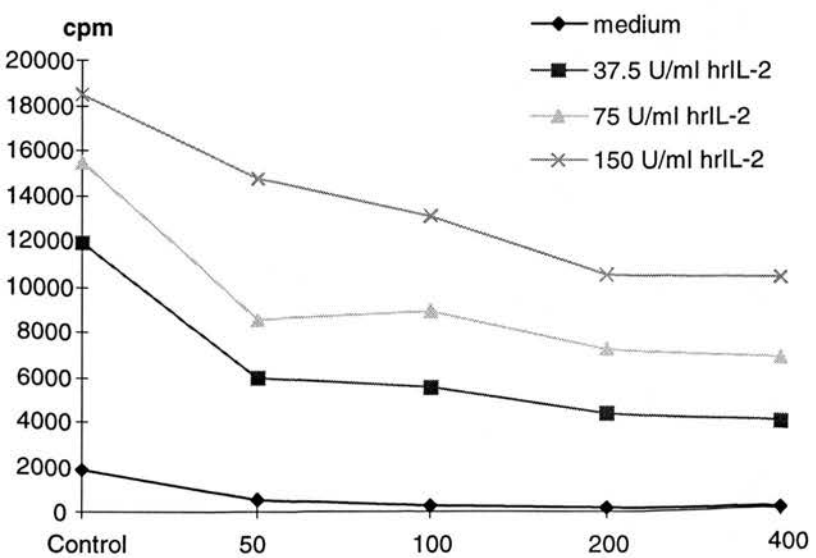
The inhibition by Cs A on all four cell lines increased significantly ($p < 0.01$) as a function of the length of the exposure and time (Figure 9.8 and 9.9).

Figure 9. 8 Mean II_{CsA} for 50 ng/ml of Cs A over *time* of OHV-2 positive cell lines cultured in medium alone



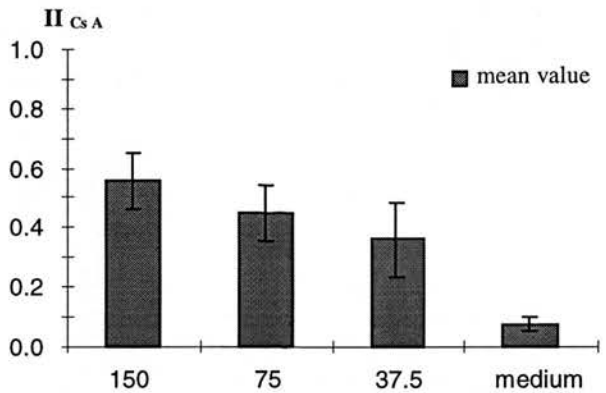
$II_{Cs\,A}$ expresses the relative inhibition by Cs A respective to the control (formula in 9.2.4)

Figure 9. 9 Thymidine uptake (mean value) by the four cell lines after 72 hours incubation with various concentrations of Cs A (sem given in table 9.6)



It was also observed that the concentrations of IL-2 had a significant ($p < 0.01$) influence on the inhibition (Figure 9.9 and 10). Whereas cells cultured with medium alone were inhibited almost completely after 72 hours by the doses of Cs A employed, cultures stimulated by IL-2 were only partially inhibited and the cpm was always higher than the medium control without IL-2 (Figure 9.10).

Figure 9. 10 Inhibition by Cs A (400 ng/ml) of four cell lines incubated for 72 hours with varying dilutions of IL-2



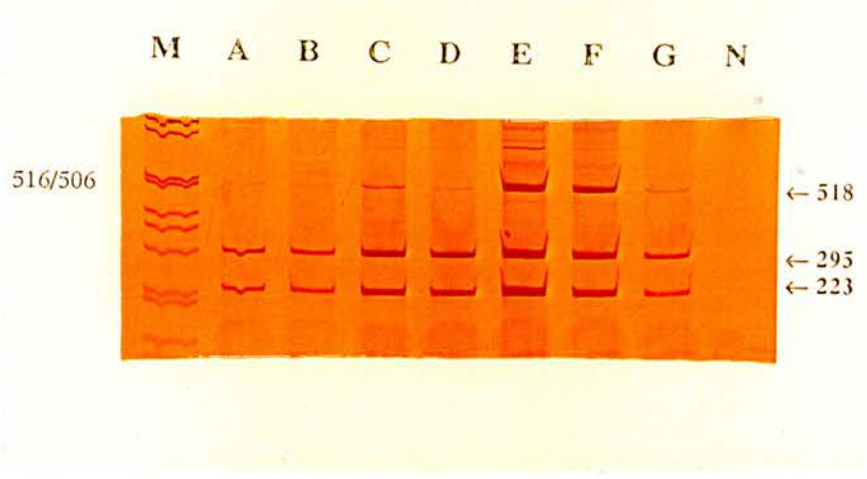
9.3.6 Effect of anti-IFNγ on OHV-2 positive cell lines

The effect of anti-IFNγ on the growth of cell line BJ 1004 was investigated, since it was shown that IFNγ was transcribed and biologically active IFNγ could be detected in the supernatant of various cell lines (Dr. H.W. Reid, personal communication). The antibody as well as the control antibody had no effect on the growth of IL-2-stimulated and unstimulated cells either at 24 hours or at 52 hours.

9.3.7 Cytokine profile

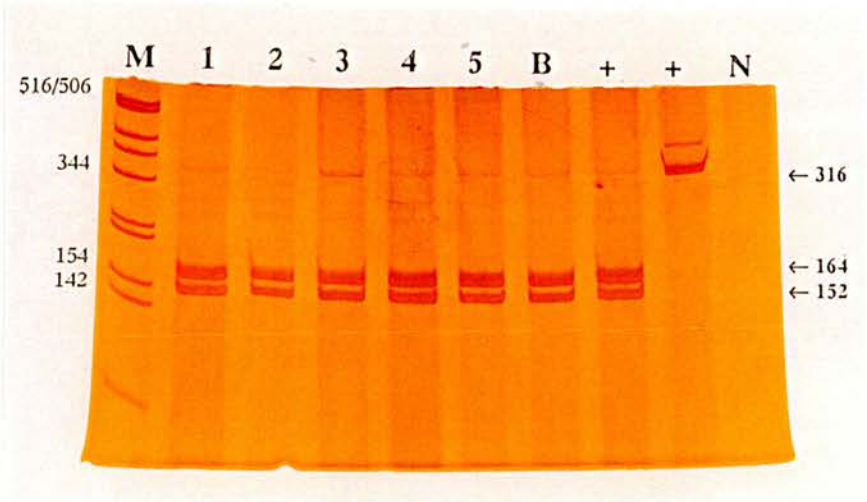
The examination of RNA derived from five OHV-2 cell lines (BJ 971, BJ 1004, BJ 1035, BJ 1044 and BJ 1104) and RNA derived from Con A blasts showed that Con A blasts transcribed all cytokines tested for whereas the cell lines transcribed IL-10 (Figure 9.11), TNFα and IFNγ (Figure 9.12), but did not transcribe IL-2 (Figure 9.13) and IL-1β. IL-4 was transcribed by three out of four cell lines. Since the PCRs were performed at the same time on the same cDNA, the selective positivity of the RT-PCR shows that the RNA was in good condition and that no genomic DNA was amplified.

Figure 9. 11 IL-10 transcription by OHV-2 positive cell lines detected by RT-PCR and RE analysis



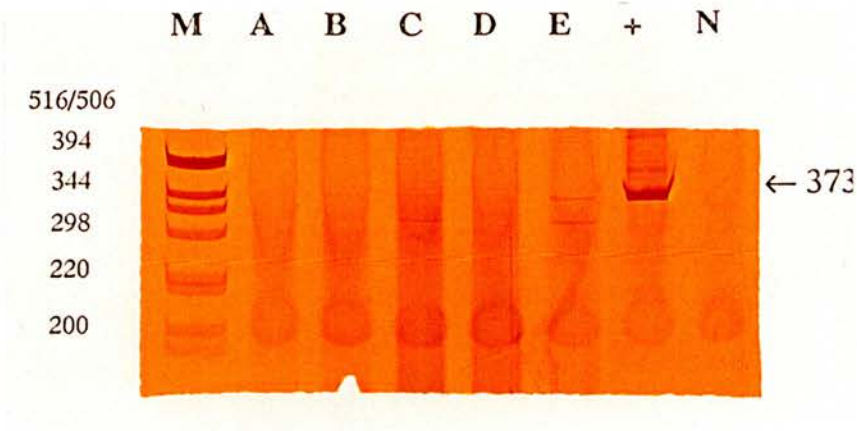
M	marker	**E**	BJ 1104
A	BJ 1004 IL-2 unstimulated	**F**	BJ 971
B	BJ 1004 IL-2 stimulated	**G**	bovine Con A blasts
C	BJ 1035	**N**	negative
D	BJ 1044		

Figure 9. 12 INFγ transcription by OHV-2 positive cell lines detected by RT-PCR and RE analysis



M	marker	5	BJ 1104
1	BJ 971	B	bovine Con A blasts
2	BJ 1004	+	positive clone (cut and uncut)
3	BJ 1035		
4	BJ 1044	N	negative

Figure 9. 13 IL-2 transcription by OHV-2 positive cell lines detected by RT-PCR amplified with the primers designed by Dr. R. Collins



M	marker	D	BJ 1044
A	BJ 971	E	BJ 1104
B	BJ 1004	+	bovine Con A blasts
C	BJ 1035	N	negative

9.4 Discussion

IL-2 dependent cell lines which retained the OHV-2 genome were established from lymph nodes as well as cornea and CSF as described previously by other authors (Chapter 1.2.6.2). The phenotypic analysis of the IL-2 dependent cell lines confirmed the finding of Burrells *et al.,* (1991), that these cell lines derived from SA-MCF affected cattle are T-cells even though it was not always possible to detect other cell surface markers such as CD4 and CD8. However two monocyte type cell lines were also established from SA-MCF-affected animals. This finding concords with the observation of Mushi and Rurangirwa (1981b), that macrophages from AHV-1 infected rabbits also retained infectivity.

The variety of cell lines which can be established could suggest that the type of cell harbouring OHV-2 in the affected animals may determine the clinico-pathological picture of the disease. However, since a $CD4^+$ cell line as well as a $CD8^+$ cell line and monocyte type cell lines were isolated from one animal (BJ 1004), this hypothesis seems unlikely. It appears more probable that OHV-2, like other herpesviruses, has the potential to infect a variety of cells (epithelial cells, T-cells, monocyte type cells). However, only an understanding of the mechanism(s) responsible for OHV-2 entry into the cells, whether involving complete viral particles or by direct cell-to-cell transfer through contact, will establish the full variety of cells which can be infected.

These data suggest that the immortalisation process induced by OHV-2 is independent of the phenotype of cell which is infected. It was, therefore, decided to evaluate functional characteristics of these cell lines and for simplicity, the studies concentrated on lymph node derived IL-2 dependent cell lines. Since cryopreservation was not possible and the cell lines were unpredictable in culture, several cell lines were lost particularly at the beginning of the study. It was, therefore, not possible to perform all the experiments on all cell lines.

9.4.1 Do continuously growing T-cell lines derived from SA-MCF affected cattle produce IL-2?

With regard to the initial hypothesis of this study, that MCF inducing viruses provoke hyperproduction of IL-2, the role of IL-2 in OHV-2 positive cell lines was examined. The specificity of the IL-2 dependency of these cell lines was confirmed since cells reacted to human as well as bovine rIL-2. That bovine IL-2 was more effective in stimulating the cells than human IL-2 was expected since homologous systems are more specific. Furthermore, this reactivity was mediated through the IL-2Rα unit as shown by neutralising the response to IL-2 with anti-bovine CD25 specific as far as is known only for this cytokine (Naessens *et al.,* 1992). The labelling with anti-bovine CD25 of the cell lines further underlined these findings.

The need for exogenous IL-2 for the continuous growth of OHV-2 T-cell lines indicates that these cells do not produce their own IL-2. That these cell lines are not transcribing IL-2 was confirmed by the absence of detectable IL-2 transcripts even though three different primer sets were used. These data suggest that OHV-2-harbouring cell lines are responding to IL-2 due to IL-2Rα upregulation, but that they do not produce IL-2 as T-cells do upon IL-2R/IL-2 interaction (Depper *et al.,* 1985). The hypothesis was further tested by examining cell growth with a T-cell mitogen which under normal conditions induces the production of IL-2. It was shown that cells would respond to IL-2, but only moderately to Con A, a typical T-cell mitogen which induces IL-2 production through stimulation of the TCR complex. The failure to fully respond to Con A could be due to the inability to produce IL-2 and/or the lack of TCR complex and alteration of the relevant transcriptional pathway (Chapter 10.2.2).

Overall, these data show that OHV-2^+ cell lines do not transcribe IL-2, even after IL-2Rα stimulation, and that the exogenous IL-2 is necessary for continuous cell growth.

9.4.2 Is OHV-2 influencing the transcription of other cytokines?

The observations that the establishment of OHV-2-positive cell lines was cell density dependent and that total medium exchange resulted in cell death, led to the hypothesis that other mediator/s could be involved in the continuous growth of these cell lines. The fact that the stimulation of the IL-2R triggers a variety of effects apart from mitosis further strengthens this assumption (Chapter 1.3.1). Similar observations were made with other IL-2 dependent continuously growing cell lines induced by a variety of pathogens which all generate lymphoproliferative diseases and transformation of CD4 as well as CD8 positive T-cells such as HVS (reviewed by Mittrückner *et al.,* 1992), *Theileria parva* (reviewed by Eichhorn and Doebbelaere, 1994) and HTLV-1 (reviewed by Rohwer *et al.,* 1994).

To test if an immunologically active protein could be involved in the mechanism of continuous growth, the effect of Cs A, as an inhibitor of NF-ATp, was assessed. The results show that Cs A inhibits cell lines in a time and dose dependent manner. Since IL-2 did partially overcome this inhibitory effect, it can be presumed that the binding of IL-2 to its receptor complex and the resulting continuous growth is due to at least two distinct events:

1. direct induction of mitosis
2. induction of Cs A sensitive factor/s

Whereas the induction of mitosis is a well known phenomenon after IL-2R binding (Chapter 1.3.1), the second event can be due to a variety of factors which lead to further cell multiplication. Cs A inhibits transcription of a variety of cytokine/receptor systems (Chapter 9.1.6), which could act synergistically with IL-2. Furthermore, Cs A could alter the expression of IL-2R. It is not evident from the literature if Cs A inhibits IL-2Rα expression since the data published on this topic are contradictory. In addition, Cs A inhibits another cytokine promoter, NF-κB, through a TNFα mediated mechanism (Foxwell *et al.,* 1990, 1993; Yoshimura *et al.,* 1991; Pimental-Muinos *et al.,* 1994; Rebollo *et al.,* 1995).

A further mechanism responsible for the Cs A induced inhibition would be the presence of a similar promoter region in the viral genome and, therefore, Cs A would interrupt the transcription of potential transforming protein/s. Since the immunosuppressant effect of Cs A is not fully understood other mechanisms could be involved in the observed inhibition and it is likely that, in the case of OHV-2-positive cell lines, different mechanisms are implicated.

It was, therefore, decided to examine the cell lines for the presence of a variety of cytokines involved in different phases of the normal antigen driven immune response. Preliminary experiments in which condition medium from these cell lines was titrated onto PBL from healthy cattle gave only inconsistent results and it was, therefore, decided to establish a cytokine profile by detection of mRNA. The results show that all cell lines constitutively transcribe IL-10, TNFα and IFNγ and four out of five cell lines transcribe IL-4 mRNA. IL-1β and, as discussed above, IL-2 transcripts could not be detected. This cytokine pattern observed has not previously been described in a single cell type either in the mouse model, or in attempts to classify clones from other species (Table 9.7).

Table 9. 7 Comparison of the cytokine profile of OHV-2-positive cell lines with $CD8^+$ and $CD4^+$ cell clones of other species

Cytokine	*OHV-2* *$CD4^+$ or $CD8^+$*	$CD4^+$ Th_1 [2]	$CD4^+$ Th_2 [2]	bov $CD8^+$ clones [1]	$CD8^+$ type 1 [3]	$CD8^+$ type 2 [3]
IL-1β	neg	*nd*	*nd*	*nd*	*nd*	*nd*
IL-2	neg	+	neg	+	+	+
IL-4	+ (4/5)	neg	+	+	neg	+
IL-10	+	neg (+ bov, hum)	+	+	neg	+
TNFα	+	+	+	*nd*	+	+
IFNγ	+	+	neg	+	+	+

[1] Con A derived bovine $CD8^+$ clones with unknown antigen specificity (Brown *et al.*, 1994); [2] classification of mouse helper cells (adapted from Annie *et al.*, 1990) very similar clones exist for human (Salgame *et al.*, 1991) and bovine clones (Brown *et al.*, 1994) with the difference that the clones are producing IL-10; [3] $CD8^+$ clones derived from humans with leprosy (Salgame *et al.*, 1991); **neg** negative; ***nd*** not described. Filled boxes denote similar pattern

Since no difference in cytokine pattern was detected between cell lines of different phenotype and since IL-10 and IL-4 physiologically down-regulate TNFα and IFNγ (Table 9.1), it is possible that OHV-2 is interfering with the transcription of various cytokines. The evaluation of human $CD4^+$ T-cell lines transformed *in vitro* by HVS showed that Th_1 clones retained their pattern of cytokine secretion, whereas the profile of Th_2 cells was modulated towards a Th_1 response with induction of IFNγ and diminished production of IL-4 (De Carli *et al.,* 1993). It could, therefore, be hypothesised that OHV-2 influences cells in a similar manner to HVS. Further work should be performed on possible alteration by OHV-2 of the expression of the IL-2, IL-4, IL-10, TNFα and IFNγ cytokine receptor by OHV-2-positive cell lines as well as of signal transductional pathways.

Since it is not possible to clone OHV-2-positive T-cell lines, it might also be presumed that a variety of cell types are present and that the different cell types are producing different cytokines. This hypothesis can partially be ruled out since cell line BJ 971 and BJ 1035 were 99.5 % and 99.6% CD8 positive respectively, whereas cell line BJ 1004 was 91.8% CD4 positive when examined.

9.4.3 Biological significance of cytokine induction by OHV-2 *in vivo*

The observation that IL-4, IL-10, IFNγ and TNFα are transcribed by OHV-2-positive cell lines could suggest that these cytokines might be crucial for the development of MCF *in vivo*. Since it was shown that Cs A inhibited the T-cell multiplication *in vivo*, but did not alter the lethal course of the disease in OHV-2 infected rabbits (Buxton *et al.,* 1984), it might be assumed that in cattle OHV-2-infected cytokine-secreting lymphocytes, even if present in a low number, induce the observed pathology.

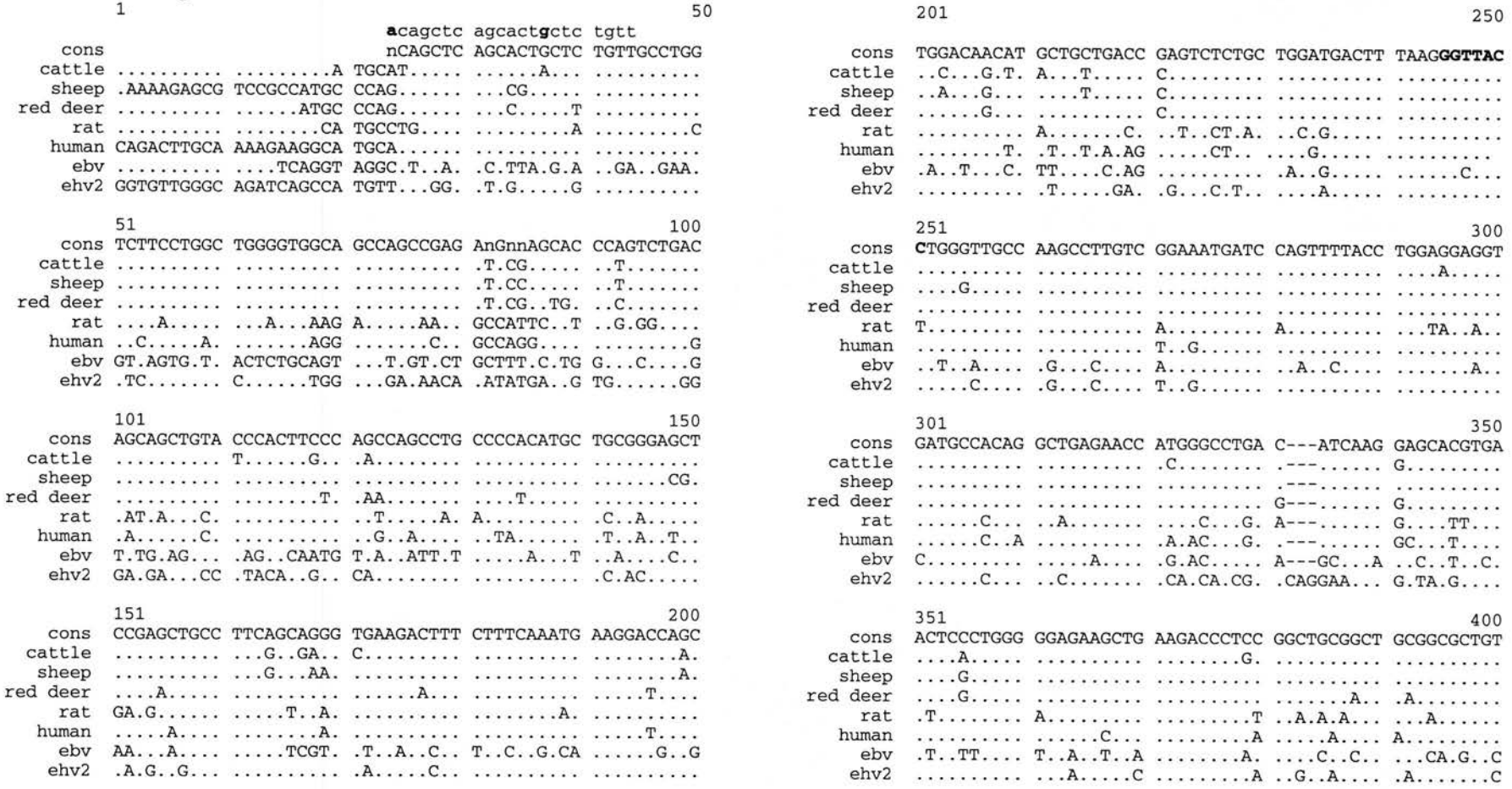
TNFα and IFNγ, as proinflammatory cytokines could be involved in the establishment of the febrile phase of MCF, whereas the role of IL-4, as a antagonist to IL-2 in cattle, is difficult to establish. The potential role of IL-10 is more complex since it is mostly an inhibitory factor in the cytokine network (Chapter 9.1.3). It also has to be questioned if this cytokine is of host or viral derivation. The viral IL-10 homologues (vIL-10) of EBV and EHV-2 are closely related to the host IL-10 on genomic (Figure 9.14) as well as biological criteria (reviewed by Mosmann, 1994).

Since the annealing temperature used for the PCR was not very stringent and the restriction enzyme does not discriminate between vIL-10 and IL-10, the derivation of the amplified product will need further study (Figure 9.14).

The role of IL-10 during EBV induced disease is not fully understood, but it has been shown that vIL-10 is expressed during the lytic (productive phase) of EBV infection (Stewart *et al.,* 1994). Furthermore, human IL-10 inhibited apoptosis of T-cells derived from human mononucleosis patients which consequently proliferated in response to IL-2 (Taga *et al.,* 1994). This fact is in concordance with the finding of others that IL-10 increases the expression of IL-2Rα on B as well as T-cells (Fluckiger *et al.,* 1993; Cohen *et al.,* 1994).

Since the four cytokines detected are antagonists during the physiological immune response and the cytokine repertoire was shown to be independent from the CD4/CD8 phenotype, the role of these cytokines *in vivo* must be carefully examined in future work.

Figure 9. 14 Comparison of viral and mammalian IL-10 nucleic acid sequences *



```
       401                                                        450
    cons  CATCGATTTC TGCCCTGCGA AAACAAGAGC AAGGCGGTGG AGCAGGTGAA
  cattle  .....C.... .......... .......... .......... ..A.......
   sheep  .....T.... .......... .......... .......... ..........
red deer  .....C.... .......... .......... .......... ..........
     rat  .......... .C.....T.. G..T..A... .....A.... ..........
   human  .......... .T.....T.. .......... .....C.... ..........
     ebv  ..CA.G..C. ....G..T.. G........T ..A..T.... .A...A.A..
    ehv2  ..CA....C. .......... G..T...... .....C.... ..........

       451                                                        500
    cons  GAGTGTCTTC AATAAGCTCC AAGAGAAAGG TGTCTACAAA GCCATGAGTG
  cattle  ...A...... .G.G...... ......GG.. .......... ..........
   sheep  ...A...... ....T..... ......GG.. .......... ..........
red deer  ...C...... .G........ ......GG.. .......... ..........
     rat  ..A..AT..T .......... ....C..... .........G .......A..
   human  ..A..C...T .......... .......... CA........ ..........
     ebv  A.A..C...T ..C.....G. .G..A..... AA.T...... ..........
    ehv2  ...C.C.... .GC....... .G........ G........G ........C.

       501                                                        550
                                        catagaat cctacatgac aacg
    cons  AGTTTGACAT CTTCATCAAC TACATAGAAG CCTACATGAC AATGAAGAT-
  cattle  .......... .......... .........A .......... ..C......-
   sheep  .......... .......... .........T .......... ..C......-
red deer  .......... .......... .........A ......C... ..........-
     rat  .......... .......... .......... .....G.... .C.C..A..G
   human  .......... .......... .......... .......... .........A
     ebv  .A........ T..T..T... .......... .A........ ...T..AGCC
    ehv2  .......... ..T...T... .......... .......... C.CA.....G
```

* primers are shown in the top row whereas the restriction site is highlighted in bold

9.4.4 Potential role of CD2

CD2 is a cell surface protein which functions as an adhesion molecule to vascular endothelium and other lymphocytes as well as a co-stimulatory molecule during T-cell activation. It is detectable on T-cell as well as NK-cells and is responsible for the agglutination of T-cells with sheep red blood cells. CD2 is the receptor for CD58 (LFA-3) in human and CD48 in rat and mouse. The stimulation of CD2, in context with the TCR and other costimulatory molecules, increases the tyrosine kinase activity of P56 (lck) and P59 (fyn), enzymes important for the signal transductional pathway involved in T-cell activation. Their activation induces the expression of IL-2Rα (reviewed by Bell and Imboden, 1995).

The difficulty of establishing and maintaining OHV-2 cell lines at low density suggests that cell to cell contact is necessary for continuous growth. Since the cells are always highly positive for CD2 even in the absence of other lymphocyte surface antigens, it might be possible that cells auto-stimulate each other by CD2/bovine equivalent to LFA-3 (bovLFA-3) interaction. Similar data were reported for HVS transformed human T-lymphocytes which proliferate in response to CD2/LFA-3 interaction in an IL-2 dependent manner (Mittrückner *et al.,* 1992). It was further observed that the change in cytokine pattern observed in HVS transformed T-cell lines is enhanced after CD2 stimulation (De Carli *et al.,* 1993). Furthermore, it has been shown that CD2 functions as a regulator of these anergic T-cell clones (Chapter 10.2).

Additionally, the stimulation via CD2 of human peripheral T-cells induces long term autocrine proliferation through the activation of NF-κB (Costello *et al.,* 1993). The cytokines involved in this process were mainly IL-10, but also IL-4 and INFγ, whereas IL-2 was induced only to a very low degree (Schwarz *et al.,* 1995). This observation would, therefore, indicate that the cytokine profile is mainly due to the autostimulatory effect through CD2 and not induced by OHV-2.

Future work should, therefore, include the investigation of the role of CD2 during the immortalisation process. Continuous stimulation with CD2 might open the possibility to clone the cell lines and would, therefore, assist with the understanding

of signal transduction in cattle and also with identification of the role of OHV-2 in these cells.

9.5 Summary

A variety of cell lines, containing viral genome, were established from SA-MCF affected cattle. Two cell lines were of a monocytic phenotype, whereas all others were large granular lymphocyte type cells. The characterisation of these large granular lymphocytes showed that

1. they constitutively express CD2 and CD5, whereas the expression of CD4 and CD8 was variable, but negatively correlated
2. the continuous maintenance of cell lines was contact, supernatant, and IL-2 dependent
3. the activity of IL-2 was mediated through the IL-2Rα
4. these cells reacted only poorly to Con A
5. Cs A inhibits the growth in a time and dose-specific manner even though IL-2 partially overcame this inhibition
6. cell lines did not transcribe IL-2 and IL-1β
7. cell lines constitutively transcribed IL-10, TNFα and IFNγ, most cell lines transcribed IL-4

Chapter 10

General Discussion

10.1 Introduction

The initial hypothesis of this study was based on the assumption that the lymphoid cell hyperplasia observed in MCF occurs through a virus induced hyperproduction of IL-2, a cytokine known for its potential to induce T-cell multiplication (Chapter 1.2). The hypothesis was tested in the rabbit model with AHV-1 as well as *in vitro* with OHV-2 by characterising the effects induced by these viruses using histological, immunocytochemical, molecular biological and cell culture techniques. The specific results and the technical problems encountered have already been discussed in the appropriate chapters. This general discussion will therefore focus on the possible role of IL-2 in the pathogenesis of SA- and WA-MCF and consider pathogenetic mechanisms which should be the focus of future work.

For this discussion, it is essential to distinguish three pathogenetic events:

1. primary effect of the virus on the cell
2. secondary effect of the infected cell on the regulation of uninfected lymphoid cells
3. physiological response to the presence of foreign antigen (virus), cell death and haemorrhages

The results obtained from the characterisation of cell lines derived from SA-MCF affected cattle provide the basis for the further understanding of the effect of OHV-2 on the single cell. In contrast, the experiments performed with AHV-1 infected rabbits describe the effect of this virus on the whole organism. Even though it always must be remembered that two distinct viruses have been investigated in two different systems, there are certain conclusions that can be drawn.

10.2 Role of IL-2 in the pathogenesis of MCF

The histological examination of rabbits experimentally infected with AHV-1, OHV-2 and HipHV-1 confirmed the findings of other authors that the aetiological agents of MCF induce a lymphoproliferative disease comparable to the disease found in cattle (Chapter 3 and 8). Though differences in the distribution pattern of lymphoid cell

hyperplasia were detected with the different viruses, the fundamental effect on lymphoid organs and T-cells in non-lymphoid organs appears to be the same. As has been stressed many times before, virus, even though present, is difficult to detect in affected animals (Chapter 1). It was therefore hypothesised that the three viruses studied induce an upregulation of IL-2 and that the hyperplasia is the pathological consequence of this.

This study showed that OHV-2 positive cell lines transcribe a well defined set of cytokines. The most important observation in this context was that IL-2 transcription was not detected in these cells. In contrast, it was shown that IL-2 was transcribed in spleen and lymph nodes during the acute phase of the disease in AHV-1 infected rabbits. However, very little IL-2 activity was detected in supernatants from short term cultures derived from various organs from these animals.

If IL-2 transcription is upregulated by these herpesviruses, IL-2 mRNA should have been identified in OHV-2 cell lines and excess IL-2 should also have been detected in the supernatants of short term cultures of cells derived from AHV-1 infected rabbits. The finding that IL-2 transcripts are present in splenocytes and lymph node cells during the acute phase of the disease, can therefore be interpreted as a subordinate consequence to the viral infection such as immune response against AHV-1 products. It was therefore anticipated that the amplification of mRNA derived from lymph node cell populations from SA-MCF affected cattle would reveal IL-2 transcripts. It was, however, considered that such a result would not have answered any crucial question, and thus the experiment was not performed.

That T-lymphocyte hyperplasia observed in MCF could occur in the absence of IL-2 is supported by the observations that in transgenic mice and in patients with IL-2 dependent immunodeficiency, T-cell maturation as well as T-cell activity is not compromised (Chapter 1.3.3)

It is therefore concluded that transcription of IL-2 is not a critical event in the pathogenesis of MCF.

10.3 Possible mechanisms involved in the pathogenesis of MCF

Though the initial hypothesis of this study could not be confirmed, the experiments lead to several interesting observations which are summarised as follows (Table 10:1).

The initial comparative studies of **rabbits** infected with OHV-2, AHV-1 and HipHV-1 demonstrated that the viruses target different compartments of the lymphoid system to a varying degree (Chapter 3). The phenotypic analysis showed that $CD43^+$ T-cells predominantly multiply *in situ* (Chapter 4). The proportion of various lymphocyte subpopulations in the spleen and lymph nodes was not altered, whereas $CD5^+$ and $CD8^+$ cells were enlarged with respect to the controls (Chapter 4). The lymphoid cell hyperplasia was further demonstrated by the observation that lymphocytes derived from MCF affected lymph nodes have a high thymidine uptake in the first 24 hours compared to the controls. The explanted cells reacted to exogenous IL-2 and weakly to Con A (chapter 5). IL-2 mRNA could be detected in these cells (Chapter 7) and very little IL-2 activity was found in the supernatants derived from short term cultures (Chapter 6).

The characterisation of the IL-2 dependent **cell lines** harbouring the OHV-2 genome showed that they are of variable phenotype and do not transcribe IL-2 or IL-1β. It was, furthermore, shown that they react weakly to Con A, are inhibited by Cyclosporin A and transcribe IL-4, IL-10, TNFα and INFγ (Chapter 9).

These data are discussed with respect to the possible primary target cells for MCF inducing viruses. Furthermore, the possible role of the IL-2R in the pathogenesis is considered.

Table 10. 1 Summary of the results obtained

Parameter	Cell line primary effect	Rabbit [1] secondary effect
Virus	OHV-2	AHV-1
lymphoid cell hyperplasia	continuous growth	*in situ* multiplication of lymphocytes in lymphoid and non lymphoid tissues
phenotype	$CD2^+CD5^+$ cell lines with variable CD4/CD8 phenotype	all cell types are involved, but predominantly T-cells
IL-2 activity in supernatants	negative	relatively low
IL-2 mRNA	negative	+
Response to hrIL-2	+	+
Con A reactivity	weak	weak
Cyclosporin A	sensitive	(sensitive [2])
Other cytokines	IL-4, IL-10, TNFα, IFNγ	**?**

[1] pathological examination and short term cultures of lymphocytes derived from rabbits during the febrile phase of WA-MCF

[2] work carried out by Buxton *et al.* (1984) showed that the T-cell hyperplasia in rabbits is inhibited by Cs A

10.3.1 Target cells of MCF inducing viruses

Lymphocyte homing involves activation of a variable combination of receptor-ligand interactions on endothelial cells as well as circulating lymphocytes. A series of differing activation mechanisms are responsible for the regulation of these ligands such as antigen activation, chemokines (IL-8, MIP-1α, etc.) and cytokines (IL-1, TNF α and IL-4) (for review see: Springer 1990; Shimizu *et al.* 1992; Pardi 1992; Picker, 1994) (Figure 10.1). It has been hypothesised that herpesviruses can interfere with lymphocyte recruitment, since viral sequences which are homologues of chemokines and chemokine receptors were recently identified for HVS, human Cytomegalovirus and HHV-6 (Chapter 1.4.2).

The finding that OHV-2, AHV-1 and HipHV-1 induce a **different distribution pattern** of hyperplastic lesions is indicative of a peripheral lymphocyte dysregulation which affects different compartments of the immune system to a varying degree and therefore may involve the regulation of adhesion molecules (Chapter 3 and 4). Reid and Buxton (1989) proposed, that the viruses infect distinct lymphocyte subsets which are characterised by tissue-specific adhesion molecules or alternatively in endothelial cells. The infected **lymphocytes** would therefore home in a specific compartment of the lymphoid system and initiate the characteristic lymphoid cell hyperplasia(Figure 10:1-**1**).

The hyperplasia of distinct lymph nodes and the accumulation of $CD43^+$ T-cells (Chapter 3 and 4) could also be the consequence of direct (intracellular) or indirect (extracellular) interference with the regulation of inducible adhesion molecules. The viruses could infect **endothelial cells or lymphocytes** and thereby interfere with the expression of adhesion molecules (Figure 10:1-**2**). However, the adhesion molecules could also be induced indirectly by cytokines and chemokines important for lymphocyte recruitment (Figure 10:1-**3**). The cytokine cascade responsible for the lymphocyte accumulations could be initiated by **resident cells** such as γ/δ T-cells, macrophages and other types of antigen presenting cells which might be the primary target cell for these viruses *in vivo*. The observation that macrophage type cells can also carry viral genome would support this hypothesis as well as explaining the low frequency of virus positive cells in MCF-affected animals. The sensitivity to

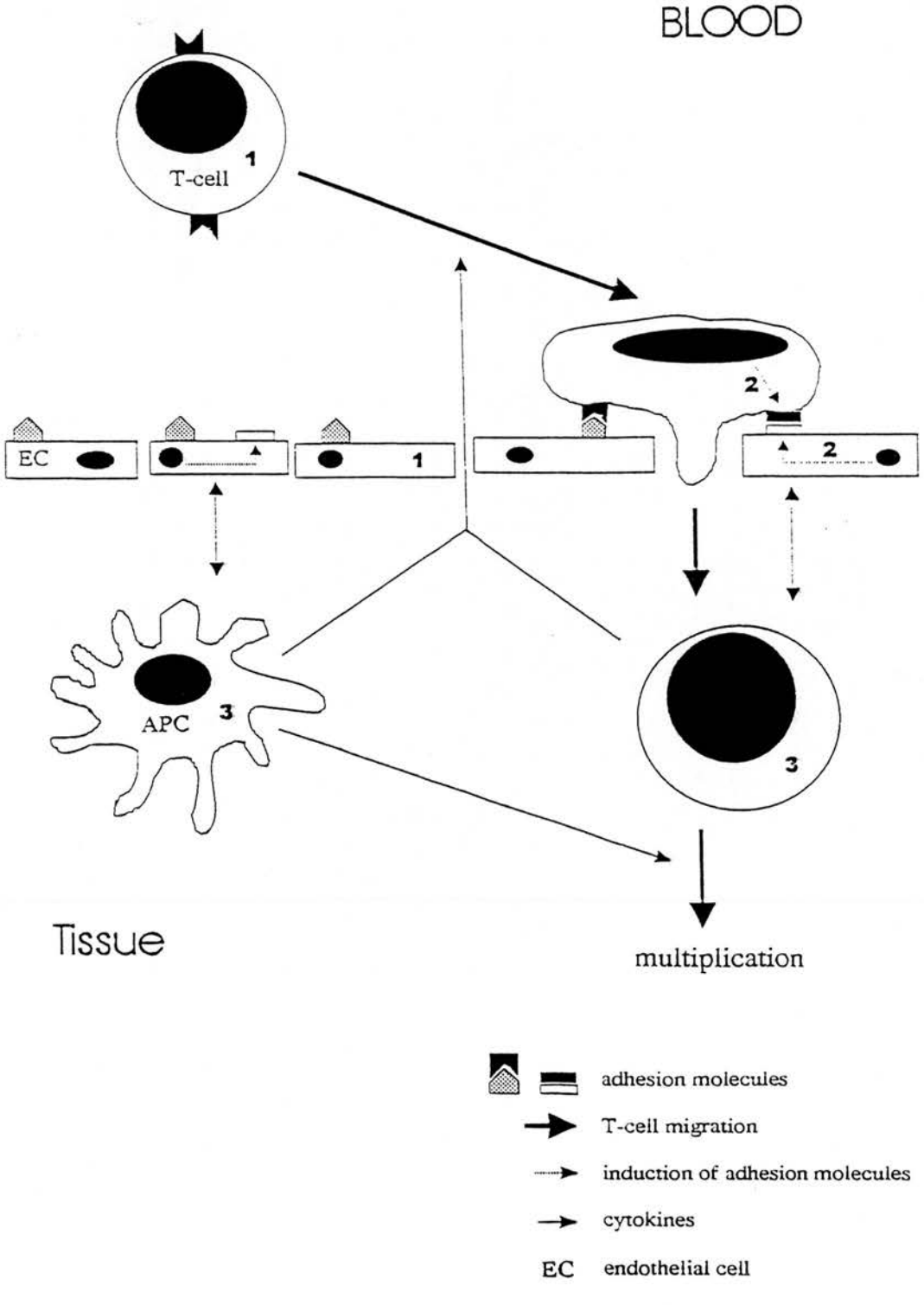
Cyclosporin A and the uniform cytokine pattern observed in OHV-2 lymphoblastoid cell lines which is independent of their phenotype, suggests that OHV-2 is able to interfere with cytokine transcription (Chapter 9). This observation favours the hypothesis that OHV-2 could interfere indirectly with the regulation of lymphocyte homing.

One interesting hypothesis is that endothelial cells, acting as non conventional antigen presenting cells might be infected by OHV-2 and could be responsible for the vascular lesions observed in MCF (O'Toole *et al.* 1995).

Since T-cell lines which contain viral genome can be generated from MCF affected animals, it can be presumed that cells of the immune system and specifically **T-cells** are the main target cells for OHV-2 and AHV-1 *in vivo* (Chapter 1.1 and 9). Both herpesviruses have the capacity to induce continuous growth of T-cells of variable CD4/CD8 phenotype in a similar fashion to the T-lymphotropic herpesviruses HVS and Marek Disease Virus (MDV) (Mittrückner *et al.* 1992; Schat *et al.* 1991). It is not known if OHV-2 and AHV-1 DNA is present in these cell lines in an **episomal form** as described for HVS transformed cell lines (Simmer *et al.* 1991) or if the genome is **integrated** as in the cell lines induced by MDV (Delecluse *et al.* 1993). The form in which the virus is present in the T-cell lines will have implications for the pathogenetic mechanism of the disease in as much as the regulation and interference of viral as well as host transcription will differ dependent on the physical state of the viral DNA. The frequency of OHV-2 positive clones derived from a genomic library of one cell line suggested that multiple viral copies were present in each cell supporting the view that the viral DNA was present in episomal form (Bridgen and Reid, 1989).

The primary target cell *in vivo* has not been identified as yet. A possible way which would allow the identification of this initial step in the pathogenesis of MCF would be comparative studies of adhesion molecules during infection with AHV-1, OHV-2 and HipHV-1. Additionally, the further characterisation of lymphoblastoid cell lines would give evidence of the molecular interaction which the viruses might have at the transcriptional level and reveal possible indirect mechanisms.

Figure 10. 1 Mechanisms involved in lymphocyte homing which might play a role in the different distribution pattern observed in AHV-1, HipHV-1 and OHV-2 induced MCF of rabbits (1- infection of lymphocytes or endothelial cells expressing tissue-specific adhesion molecules; 2- interference by the virus with the transcription of inducible adhesion molecules; 3- infection of resident cells which induce the cell accumulation via cytokine induction)



10.3.2 The role of the IL-2R during infection with AHV-1 and OHV-2

IL-2Rα is not expressed on resting T-cells and can only be detected on the cell surface after antigen or mitogen activation. Once the stimulus ceases, the receptor is down-regulated by intra/intercellular mechanisms important for the termination of the normal immune response (reviewed by Smith, 1988). It was shown that abnormal expression of IL-2R is associated with severe pathology in immunological disorders such as certain forms of leukaemia (Adult T-cell leukaemia, cutaneous T-cell lymphoma and Hodgkin's disease), autoimmune disease (systemic lupus erythematosus, sarcoidosis, non infectious uveitis) and graft versus host disease (reviewed by Waldmann, 1993).

Evidence described in this study suggests that IL-2 induction is not a crucial step in the pathogenesis of MCF, whereas the possibility that its receptor might be upregulated due to the viral infection could not be excluded. Increase of IL-2R expression during MCF is suggested by the observations that OHV-2 cell lines were IL-2Rα positive and reacted to bovine as well as human rIL-2 through the IL-2Rα unit. Furthermore, freshly explanted lymph node cells derived from AHV-1 infected rabbits as well as SA-MCF affected cattle responded immediately to exogenous IL-2 indicating the presence of functional IL-2R. In addition, it was hypothesised that the failure to detect IL-2 activity in short term cultures from AHV-1 infected rabbits in the presence of IL-2 mRNA was due to a disproportionate increase of IL-2R expression and therefore binding of free IL-2.

These data related to involvement of peripheral T-cells and IL-2R expression suggest two possible pathogenetic mechanisms for MCF which are not mutually exclusive. The first mechanism would implicate alteration of IL-2R expression by a similar mechanism to that of the signal transduction pathways induced by Human T-cell leukaemia virus-1 (HTLV-1) whereas the second mechanism would be based on the assumption that normally anergic lymphocytes are activated during AHV-1 and OHV-2 infection analogous to models for autoimmune disease.

I. Possible similarities in pathogenetic mechanisms between HTLV-1 and OHV-2/AHV-1 induced disease

Human T-cell leukaemia virus-1 (HTLV-1), a human retrovirus, causes constitutive overexpression of IL-2Rα, whereas IL-2 expression seems not to be involved in the pathogenesis of the disease (reviewed by Yodoi and Uchiyama, 1992). Interestingly, adult T-cell leukaemia (ATL) induced by random integration of HTLV-1 proviral genome into the human genome is characterised by the appearance of morphologically unique T-cells in peripheral blood and lymph nodes, hepatosplenomegaly, and skin infiltration with atypical T-lymphocytes. Other syndromes caused by this virus are chronic arthropathy, uveitis, bronchopneumopathy and tropical spastic paraparesis all associated with polyclonal T-cell proliferation. The T-cells involved are of variable CD4/CD8 phenotype (Sawasdikosol *et al.* 1993; Kondo *et al.* 1995). The absence of viral oncogenes in the HTLV-1 genome led to the conclusion that the virus interacts with intermediate cellular transcription factors. It is thought that the IL-2Rα chain expression is an indication of the activation state of HTLV-1 transformed T-cell lines, some of which are IL-2 dependent and constitutively express various cytokine genes. It has been suggested that a viral gene (pX^{TAX}) interferes with the transduction pathways at the level of NF-κB, an inductional factor for IL-2Rα as well as a variety of cytokines (Ballard *et al.* 1988; Leung and Natal, 1988). Another viral induced factor, ATL-derived factor or human thioredoxin homologue, interferes with a variety of cellular functions such as cytokine induction and IL-2Rα expression (Yodoi and Uchiyama, 1992). Moreover, it has been shown that the expression of this factor is also enhanced in EBV-infected lymphoblastoid cell lines (Wakasugi *et al.* 1990; Makino *et al.* 1992).

The similarities of the target organs (lymph nodes, skin, articulations, eye), the T-cell involvement and the characteristics of the derived T-cell lines between MCF affected animals and HTLV-1 induced diseases could be an indication for a similar pathogenetic mechanism in the two different viral infections. The hypothesis of IL-2R overexpression during MCF should therefore be further tested *in vivo* and *in vitro*.

Additionally, the exact role of OHV-2/AHV-1 in the continuously growing T-cell lines should be identified quantitatively as well as qualitatively and the presumptive upregulation of IL-2R transcription investigated. Furthermore, it has still to be established, if OHV-2 really transforms these cells (expression of oncogenes and consequently immortalisation) or if the exogenous IL-2 and resultant CD2 stimulation just maintain the viability of those cell lines incidentally harbouring OHV-2.

II. Possible role of anergic cells in the pathogenesis of MCF

Tolerance is a phenomenon whereby lymphocytes are non-responsive to a given antigen. Its major implications are found in the inhibition of hyper-reactive T-cells to antigens (self and non-self) and in graft versus host disease (GvHD) (reviewed by Kappler *et al.* 1987; Kisielow *et al.* 1988; Ramasee *et al.* 1989; Miller and Morahan, 1992). Immunological tolerance is predominantly the consequence of maturation and negative selection of T-cells in the thymus. There are, however, numerous instances where this mechanism alone is not sufficient and peripheral mechanisms are required to maintain unresponsiveness in mature T-cells. One such mechanism by which the unresponsiveness is ensured in the periphery is known as clonal anergy.

Clonal anergy was first observed *in vitro* where it was shown that the stimulation of T-cell clones via the antigen specific TCR complex in the absence of costimulatory signals leads to unresponsiveness of the clone to this specific antigen (reviewed by Schwartz, 1990; Ramsdell and Fowlkes, 1990; Yssel *et al.* 1994, Lombardi *et al.* 1994). The co-stimulatory signals are given by B7, CD40 and other molecules found on APC, and the respective T-cell receptors CD28, CTLA-4 etc. Anergised T-cells are phenotypically similar to cells responding to an immunogenic stimulus, though the TCR complex in these cells is down regulated. This diminished TCR complex expression leads also to reduced reactivity to Con A. In contrast, the IL-2Rα is upregulated in anergic T-cell clones and the clones proliferate in response to exogenous IL-2. However, when restimulated with the original antigen, these cells fail to upregulate transcription of the IL-2 gene and in consequence do not proliferate. Recently CD2 has been also shown to function as an important regulator of anergic T-cell clones. After prolonged culture in IL-2, anergic T-cell clones did not react to

other costimulatory molecules such as ICAM-1 and B-7, but did proliferate in response to IL-2 and, upon stimulation through CD2, the allo-antigenic responsiveness was restored (Boussiotis *et al.* 1994; Bell and Imboden, 1995). Furthermore, it was observed in human melanoma patients that anergic T-cells clones produced IL-10 (Becker *et al.* 1994).

In vivo, high doses of exogenous IL-2 induce autoimmune disease in various mouse models and it was hypothesised that IL-2 could reactivate anergic T-cells (Andreu-Sanchez *et al.* 1991; Gutierrez-Ramos *et al.* 1992; Jacobs *et al.* 1994). It is interesting to observe that the induced disease was characterised by lymphoproliferation in the spleen, lymph nodes and kidney as long as the thymus anlage was present. Furthermore, it has been recently shown that CTLA-4 deficient mice have splenomegaly and hyperplasia of the lymph nodes, diffuse and focal lymphocyte infiltration in heart, lung, bone marrow, liver and pancreas (Waterhouse *et al.* 1995). The lymphocytes showed increased expression of CD25, CD5 and CD28. Both CD4 and CD8 populations contained activated cells and showed no change in CD4/CD8 ratio. B-cells showed up-regulation of CD5, Fas antigen and B7-2. Purified T-cells from $CTLA^{-/-}$ mice also displayed spontaneous proliferation in medium alone, which declined after 24 hours. The authors could not establish weather CTLA-4 functions as a costimulatory molecule or as a negative regulator of T-cell activation.

Whereas clonal anergy induced by antigen stimulation in the absence of co-stimulatory molecules seems to be the major peripheral control for self tolerance (Jenkins *et al.* 1987, reviewed by Schwartz 1990, Guerder *et al.* 1994) additional mechanisms have been proposed to explain the phenomena of immunologically privileged sites such as the anterior chamber of the eye, where, under certain conditions, allografts are not rejected. Even though these mechanisms are still largely not understood, it has been shown that corneal endothelial cells of the rat suppress *in vitro* lymphocyte proliferative responses to antigens or the mitogen Con A (Obritsch *et al.* 1992; Kawashima and Gregorson 1994). Although corneal endothelial cells can be induced to express MHC class II, expression of this complex does not seem to play a role because of the inhibitory activity is neither MHC restricted nor species-specific (Obritsch *et al.* 1992). T cells activated in the presence of corneal

endothelial cells were found to up-regulate IL-2R expression and IL-2 responsiveness, but their IL-2 secretion was substantially inhibited (Kawashima *et al.* 1994). These authors concluded that two mechanisms were important for the endothelial cell induced clonal anergy of T-cells: one mechanism could be induced by membrane bound mediators whereas the other mechanism appears to involve an extracellular factor/s which has not yet been identified.

It can be concluded that the induction and regulation of anergy is dependent on costimulatory molecules, many of which also function as adhesion molecules such as CD2 and ICAM-1, as well as tissue specific molecules (eye) and soluble factors.

The comparison of cell lines derived from SA-MCF affected cattle with anergic T-cell clones shows many similarities (Table 10:2). OHV-2 positive cell lines have a variable phenotype, express IL-2Rα and CD2, proliferate in response to IL-2 and are Cs A sensitive. Furthermore, it was hypothesised that CD2 might be important for the auto-stimulation of these cells.

Table 10. 2 Comparison of OHV-2 positive cell lines with anergic T-cell clones and normal T-cells

Characteristic	OHV-2 T-cell lines[1]	anergic T-cell clones[2]	resting T-cells[3]	activated T-cells[3]
Expression of IL-2Rα	+	+	n	+
Expression of IL-2	n	n	n	+
CD 4 / CD 8 expression	variable	variable	variable	variable
Expression of NF-AT	?	+	n	+++
Expression of NF-κB	?	+	n	+++
Expression of CD 2	+	+[4]	+	+
proliferate to IL-2 *in vitro*	+++	+++	+/-	+++
IL-10 transcription	+	+[5]	n	+
response to Con A	+/-	+/-	+	+

[1] results of chapter 9; [2] Schwartz, 1990; Ramsdell and Fowlkes, 1990; Yssel *et al.* 1994, Lombardi *et al.* 1994; [3] Roitt *et al.* 1993; [4] after cultivation in IL-2, Boussiotis *et al.* 1994, Bell and Imboden, 1995; [5] Becker *et al.* 1994; + present; +++ strong response or expression; +/- weak response; ? likely since cell lines are Cyclosporin A sensitive; **n** negative

The hypothesised involvement of anergic cells in the pathogenesis of MCF is further supported by the observations that, in the rabbit model, IL-2R expression might be significantly increased (see above) and the responsiveness to Con A is decreased (Chapter 5). However, explanted lymph node cells derived from SA-MCF affected cattle responded to this mitogen. This discrepancy could be explained by the fact that, in natural cases, the virus is present in even lower quantity than in experimental systems and that the primary effect of OHV-2 on a specific lymphocyte subpopulation is masked by the physiological response of 'normal' lymphocytes.

However, the hypotheses of an autoimmune phenomenon being a component of the pathogenesis of MCF is not only based on the functional studies, but is also suggested by the histopathological changes such as hyperplasia of lymphoid organs and mononuclear lymphoid cell accumulations in various organs such as skin and kidney observed in cattle as well as in rabbits. This pathological picture matches observations on GvHD (Seemayer *et al.,* 1983), CTLA-4 deficient mice, and in IL-2 induced autoimmune disease in mice (see above). That the pathogenesis of MCF might have similarities to these immunological disorders was already hypothesised by Liggitt and DeMartini (1980b). They discussed the morphological similarities between the vasculitis observed in MCF and in rejection arteriopathy.

These observations lead to the hypotheses that the mechanisms involved in the maintenance of anergy of peripheral T-cells are in fact altered during the course of MCF directly by the virus (anergic cell as target cell of the virus) or indirectly (virus infected cell activates physiologically anergic T-cells through adhesion molecules and cytokine/s). These working hypotheses could be further tested by investigating the antigen response of cell lines derived from animals with a defined immunological background and by more detailed analysis (morphologically and functionally) of the different costimulatory molecules and TCR present on these cell lines. Of special interest would be the investigation of the cell surface markers CD2 (as suggested in chapter 9) and CTLA-4. This would not only provide new information regarding the pathogenesis of MCF, but also contribute to the further understanding of bovine immunology.

10.4 Summary

MCF is a herpesvirus-induced pathological syndrome characterised by hyperplasia of lymphoid organs, subepithelial accumulations of mononuclear lymphocytes, paracheratotic hypercheratosis of skin and pregastric tract and segmental vasculitis. The aim of this study was to characterise the lymphoproliferation observed. The phenotypic examination confirmed that actively multiplying peripheral T-cells are the main effector cells in the periphery during the development of MCF whereas FACS analysis of lymphoid organs indicated that other cell types might be also involved in this process. The role of IL-2 in the induction of this hyperplasia could not be established, but the data obtained strongly suggest that IL-2 is not the major cytokine involved in the pathogenesis. In contrast, the results indicate that a hyperexpression of IL-2R may be involved in the pathology induced by OHV-2 /AHV-1. Many similarities between MCF inducing viruses and other γ-herpesviruses, HTLV-1, *Theileria* sp. and immunological disorders (autoimmune disease and graft versus host disease) were found. Several of these disorders have similar pathological features and it is possible to establish T-cell clones or T-cell lines from affected subjects which share important features. The pathology, immunology and cell biology of these diseases seem to converge on a common fundamental pathogenetic mechanism: the activation of autoreactive T-cells and more specifically of the IL-2R molecule. For the infectious agents, interference with the transcriptional regulation of cytokines, cytokine receptors or adhesion molecules were identified. It is, therefore, proposed that the pathology of MCF is due to a dysregulation of autoreactive T-cells, and future work should consider the role of adhesion molecules (which often also have costimulatory functions), IL-2R and transcriptional pathways involved in the regulation of cytokines.

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Appendix

Work accepted for publication

The paper "Characterisation of the lymphoproliferation in rabbits experimentally affected with malignant catarrhal fever" has been accepted for publication in *Veterinary Microbiology*. Official permission has been obtained from Elsevier Science B.V.

CHARACTERISATION OF THE LYMPHOPROLIFERATION IN RABBITS EXPERIMENTALLY AFFECTED WITH MALIGNANT CATARRHAL FEVER

A. Schock and H.W. Reid

Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH

Tel: 0131 664 3262 Fax: 0131 664 8001

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Abstract

Malignant catarrhal fever (MCF) in rabbits caused by the three Herpesviruses: *Alcelaphine herpesvirus-1* (AHV-1), *Ovine herpesvirus-2* (OHV-2) and *Hippotragine herpesvirus-1* (HipHV-1) induced hyperplasia of lymphoid tissues and accumulations of mononuclear lymphoid cells in non-lymphoid tissues. However, certain lymph nodes were affected preferentially. The lymphoid cells in non-lymphoid tissues were $CD43^+$ T-cells which showed evidence of *in situ* multiplication. A more detailed phenotypic analysis of splenocytes and lymph node cells in AHV-1 infected rabbits suggested that the hyperplasia was probably due to the expansion of $CD8^+$ T-cells. On the basis of

these data and the observations of other authors, that no or very little viral expression can be detected in lesions of MCF affected animals, we propose that the pathogenesis of MCF results from a dysregulation of a secretory T-cell activator. The variable pathology induced by the three viruses may reflect a quantitative or qualitative difference in this proposed activator.

Introduction

Malignant catarrhal fever (MCF) is a lymphoproliferative syndrome of large ruminants caused by viruses belonging to bovine γ-Herpesvirinae (*Alcelaphine herpesvirus-1* (AHV-1), *Ovine herpesvirus-2* (OHV-2) and possibly others, such as *Hippotragine herpesvirus-1* (HipHV-1) (Plowright *et al.* 1960; Bridgen and Reid, 1991; Reid and Bridgen, 1991).

The characteristic lesions of MCF in cattle and rabbits, in which the disease can be experimentally reproduced, are hyperplasia of the lymphoid organs, vasculitis and interstitial accumulations of mononuclear leukocytes adjacent to epithelia of a variety of tissues (Goetze, 1930; Piercy, 1955). The examination of lymph nodes from AHV-1 affected animals revealed, that only approximately one cell in 10^5 was positive for viral antigen or DNA (Liggitt and DeMartini, 1979; Patel and Edington, 1980; Bridgen *et al.* 1992). The lack of virus or viral expression in the lesions, lead to the proposition of an indirect pathogenesis. Since T-lymphoblastoid cell lines which contain the viral genome can be propagated from MCF-affected animals, it was suggested, that the MCF-inducing viruses might in some way alter the behaviour of the T-cell population (Berrie *et al.* 1984; Cook and Splitter, 1988; Burrells and Reid, 1990).

Reid and Buxton (1989) reported a different pattern of lymph node hyperplasia in AHV-1 and OHV-2 experimentally infected rabbits. In the current study, HipHV-1 was also included in the comparison to illustrate possible differences which might lead to the identification of the basic pathological mechanisms involved in the pathogenesis of MCF. Furthermore, in rabbits experimentally infected with AHV-1, the phenotype of the cells involved and the lymphocytic hyperplasia seen in the lesions were characterised with a panel of monoclonal antibodies (mAb).

Material and Methods

Rabbits

Conventionally reared New Zealand rabbits aged 3-12 months of both sexes were infected intravenously with either cell-associated AHV-1 (strain C500) or OHV-2 (strain: 86/13) or HipHV-1 (strain: BJ 668). In each case the rectal temperature was monitored and on the second day of a febrile response the rabbits were killed and the spleen, appendix and mesenteric, popliteal and submandibular lymph nodes were removed as well as non-lymphoid tissues (trachea, lung, oesophagus, liver, kidney, lachrymal gland). For histological and immunohistochemical analysis, 3,6 and 5 rabbits were each infected with AHV-1, OHV-2 and HipHV-1 respectively. Three normal unchallenged rabbits were used as control. For analysis by fluorescent activated cell sorting (FACS), 12 AHV-1 infected and 6 healthy rabbits, previously used for serum production, were used.

Processing of the Tissue

For histological examination, formalin fixed tissues were dehydrated through graded alcohols and embedded in paraffin wax. Sections were cut and stained with haematoxylin and eosin. For the immunohistochemical analysis, tissues were processed to paraffin wax by the St. Marie method. For FACS analysis, single cell suspensions were prepared from spleen and popliteal lymph nodes. Briefly, the organ was removed, the tissue finely chopped and passed through a stainless steel sieve. The cell suspension was then washed in balanced Hank's medium supplemented with 2% foetal bovine serum (FBS), 100 IU/ml penicillin, 50 μg/ml streptomycin (Northumbria Biologicals, UK) and 500 U/ml heparin (Sigma, UK) and layered onto LYMPHOPREP (Nycomed, Oslo, Norway) in equal volumes and centrifuged at 300g for 30 min.. The interface was collected, washed three times and the cells resuspended at a concentration of $5x10^6$ cells/ml.

Monoclonal Antibodies

Histological material was examined with mAb against rabbit CD43 (L11/135), a pan-T-cell marker (Wilkinson *et al.* 1992) and anti-proliferating cell nuclear antigen (anti-PCNA, DAKO, Denmark) which functions as a co-factor for DNA polymerase delta (Linden *et al.* 1992) and is mainly present during the S phase of the cell cycle (Bolton *et al.* 1992).

For the FACS analysis, the cells were labelled with the mAb anti-rabbit CD5 (Ken 5; Serotec; Kotani *et al.* 1993), anti-rabbit CD4 (Ken 4; Kotani *et al.* 1993), anti-rabbit

CD8 (12.C7; De Smet *et al.* 1983) and the B-cell marker NRBM (Wilkinson and Gordon, unpublished data).

The mAb L11/135, Ken 4, 12C.7 and NRBM were kindly provided by Dr. Wilkinson from the Royal Collage of Surgeons in London.

Immunohistochemistry

Selected sections were dewaxed and endogenous peroxidase was blocked with 1% hydrogen peroxidase in methanol for 30 min, rinsed with washing buffer (26 mM sodium phosphate, 155 mM sodium chloride, pH 7.5, 0.45% Tween 80), non specific staining was blocked by applying heat inactivated normal goat serum (Vector, UK) at a concentration of 1:4 in wash buffer for 30 min. The excess serum was blotted using filter paper and the section incubated overnight at 4^0 C with the primary antibody diluted appropriately in high salt buffer (0.5 M NaCl, 26 mM sodium phosphate, pH 7.5). The slides were washed twice and treated with the ABC technique (Vectastain Elite ABC-Kit, Vector Laboratories, UK). The sections were counterstained with Meyer's haematoxylin and mounted using a xylene-based mountant.

FACS analysis

To the single cell suspensions derived from spleen and popliteal lymph nodes, the required primary antibody diluted in FACS medium (1% FBS, 500 IU/ml heparin (Sigma, UK), 0.1% sodium azide in PBS) was added in an equal volume and incubated

for 90 min. The cells were washed twice with FACS medium and incubated with the fluorescein isothiocynate (FITC) conjugated antibody (sheep-anti-mouse, 1:50) (Dakopatts, Denmark) for 30 min. The cells were washed a further two times, fixed in 1% paraformaldehyde and transferred to FACS-tubes (Falcon 2054, Becton Dickinson). The preparation was stored at 4^0 C in the dark and read on a Becton Dickenson Facscan (Mountain View, CA) with linear amplification for forward (FWS) and side (SSC) scatter and logarithmic amplification for FITC green fluorescence (FL-1). Lymphocytes were distinguished on the basis of FWS/SSC profile. Small and large lymphocytes were distinguished on the base of the FWS/SSC ratio.

Results

Pathological Findings

All infected rabbits became febrile after a mean incubation period of 13.6 days (SEM 1.04) and there was no difference in incubation period between any of the three groups challenged with virus. Splenomegaly was observed in all infected rabbits and, on histological examination, expansion of the periarteriolar lymphoid sheaths was detected in all cases, but it was not possible to recognise any significant differences between the three infected groups.

Rabbits infected with AHV-1 had more sever lesions in popliteal and submandibular lymph nodes, whereas OHV-2 infected rabbits had more severe lesions in the mesenteric lymph nodes. In contrast, the HipHV-1 infected rabbits showed severe lesions in both peripheral and mesenteric lymph nodes (table 1). The different

distribution pattern of the lymph node lesions was statistically significant ($p < 0.05$). The affected lymph nodes showed hyperplasia accompanied by alteration of the shape, haemorrhages, oedema and focal necrosis. Expansion of the paracortex was the predominant histological finding together with haemorrhages, oedema and focal necrosis were confirmed at histological examination.

In the appendix, hyperplasia of the T-cell areas, necrosis and heterophilic polymorphonuclear cells could be found in all OHV-2 and HipHV-1 infected rabbits, whereas only one AHV-1 infected rabbit showed slight hyperplasia of this region. No other gross lesions were observed in the infected animals. On histological examination, peribiliary and perivascular lymphoid cell accumulations were observed in the liver of all affected animals. Lymphoid cell accumulations could be observed in the kidney of one OHV-2, two HipHV-1 and all AHV-1 infected rabbits as well as in the trachea of two rabbits infected with AHV-1, in two infected with HipHV-1 and in one infected with OHV-2. The same lesion was found in the lachrymal gland where it could be detected in all animals infected with AHV-1 and two infected HipHV-1. In the oesophagus, focal subepithelial accumulations of mononuclear lymphoid cells associated with ballooning of the overlying epithelia could be detected in all rabbits. Control rabbits showed no significant lesions.

Immunohistochemistry

The T-cell dependent areas of spleen and lymph nodes were identified morphologically and confirmed by labelling with anti-CD43 mAb in control rabbits. In MCF-affected rabbits, the labelling with anti-CD43 mAb confirmed the hyperplasia of

144 the T-cell areas, suggested by morphological criteria. The labelling pattern produced
145 by anti-PCNA mAb was identical to the pattern obtained with anti-CD43 mAb in the
146 spleen and the affected lymph nodes. In control rabbits, T-cell areas did not stain with
147 anti-PCNA mAb. For further characterisation of the lymphocytes, FACS analysis was
148 performed (see below).
149 The subepithelial mononuclear lymphocytes observed histologically in liver, kidney,
150 oesophagus, trachea and lachrymal gland of MCF-affected rabbits were all anti-CD43
151 and anti-PCNA positive (Figure 1). In the liver, trachea and lachrymal gland of control
152 rabbits, only few $CD43^+$ T-cells were found close to the respective epithelia, whereas
153 in the kidney only few intravascular cells of this phenotype were detected. Single
154 $CD43^+$ cell found in the non lymphoid tissues of control rabbits did not stain with mAb
155 to PCNA.

156 *FACS analysis*

157 Phenotypic analysis of lymphoid cells in the spleens (data not shown) and popliteal
158 lymph nodes showed, that $CD43^+$ and $CD5^+$ cells were present in the same proportions
159 in the AHV-1 affected animals during the acute phase of the disease as there were in
160 control rabbits. In the popliteal lymph nodes of MCF-affected rabbits, the proportion
161 of $CD4^+$ cells was significantly ($p< 0.05$) lower than in control rabbits (table 2).
162 Although a tendency towards an increase in the proportion of $CD8^+$ cells in the
163 popliteal lymph nodes of MCF affected rabbits was detected, this was not significant
164 due to the large variance which could be the effect of the various stage of hyperplasia
165 found in experimental infection. In the spleen, the proportion of $CD4^+$ cells was

166 comparable in the two groups (*MCF-infected*: 21.0% (SEM 3.2) *Control*: 22.3%
167 (SEM 3.8) and no significant difference could be observed in $CD8^+$cells (*MCF-*
168 *infected*: 34.4 % (SEM 5. 5) *Control*: 23.6 % (SEM 7.0).
169 Investigation of the size of the lymphocytes in spleen (data not shown) and popliteal
170 lymph nodes (table 2) indicated, that $CD5^+$ and $CD8^+$ cells were significantly ($p <$
171 0.05) larger in MCF affected than in control rabbits.

172 **Discussion**

173 The study showed, that AHV-1, OHV-2 and HipHV-1 all induce similar lymphoid cell
174 hyperplasia in the rabbit, but affect different lymph nodes to a varying extent, though
175 the spleen was involved in all cases. The suggestion made by Reid and Buxton (1989),
176 that the distribution pattern of lesions varies depending on the challenge virus (AHV-
177 1 and OHV-2), is therefore confirmed.
178 The histological and immunohistochemical examination of spleen and lymph nodes
179 showed that most of the hyperplasia was due to the expansion of T-cell dependent
180 areas. These data confirm the finding by Buxton *et al.* (1984) who used polyclonal
181 anti-rabbit T-cell serum to characterise the lymphoid cells involved in the pathogenesis
182 of OHV-2 infection in rabbits. The labelling with antibodies to CD 43 and PCNA of
183 non-lymphoid tissues showed, that the lesions of MCF are characterised by actively
184 multiplying T-cells. The evidence of single $CD43^+$ cells in the liver, trachea and
185 lachrymal gland in control rabbits suggests that there exists a resident T-cell
186 population in the non lymphoid tissues, which is stimulated to multiply during the
187 course of MCF, and that only a minority of the accumulating cells might derive from

188 infiltration. If this is the case, the suggestion made by Reid and Buxton (1989), that the
189 distinct distribution patterns of the MCF lesions induced by the different viruses was
190 due to infection of a specific lymphocyte subset with different homing characteristics,
191 is not supported.
192 The phenotypic analysis of spleen and lymph node cells derived from AHV-1 infected
193 rabbits, showed an increase in size of $CD5^+$ and $CD8^+$ cells. As this technique only
194 enumerates viable cells and a large proportion of dying cells is present in the MCF
195 lesions, the data could indicate that the hyperplasia is mainly due to $CD8^+$ cells. An
196 increase in number of $CD8^+$cells has also been reported in cattle affected with sheep-
197 associated MCF (Ellis *et al.* 1992; Nakajima *et al.* 1992). Since the overall
198 proportions of viable lymphocytes are not significantly different from the control
199 rabbits, other cell types may also be implicated in the acute phase of the disease.
200 Overall these findings strengthen the hypothesis of various authors, that T-cells are
201 mainly involved in the pathogenesis (reviewed by Reid and Buxton, 1989). The
202 observed *in situ* multiplication together with low viral expression suggests, that a
203 small lymphocyte subset, or perhaps antigen presenting cells are infected by the given
204 MCF virus and consequently alter the homeostasis of the lymphoid system by
205 dysregulating a T-cell activator such as interleukin-2. The different pathological pattern
206 induced by AHV-1, OHV-2 and HipHV-1 would indicate, that these viruses are
207 interfering with the immune system in a subtly different way and that further
208 comparative studies would help to identify the mediator(s).

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Table 1 Gross Pathological Changes of Lymph Nodes in MCF-affected Rabbits

Animal	*mesenteric lymph node*	*submandibular lymph node*	*popliteal lymph node*
OHV-2			
AS 92/3	+++	-	-
AS 92/8	+++++ N, H, S	-	+
AS 92/6	+++	-	-
AS 92/14	+++ H, S	-	+
AS 92/13	++++ H, S	+	+
AS 92/12	++++ S	-	+
AHV-1			
AS 92/4	-	+++ H	+++ H
AS 92/5	-	+++ H	+++ H
AS 92/7	-	+++ H	+++ H
HipHV-1			
AS 92/1	+++	+ H	-
AS 92/2	++	+ H	-
AS 92/9	+++++ N, H, S	+ H	+ H
AS 92/10	+++++ N, H, S	+ H	+ H
AS 92/11	+++ N, H, S	++ H	++ H

OHV-2	Ovine Herpesvirus-2
AHV-1	Alcelaphine Herpesvirus-1
HipHV-1	Hippotragine Herpesvirus-1
+	relative increase in size
N	necrosis
H	haemorrhages
S	alteration in shape

Table 2 Phenotype of leukocytes derived from the popliteal lymph nodes of rabbits affected with MCF induced by AHV-1

mAb	*MCF-infected*		*Control*	
	%	% large cells[3]	%	% large cells[3]
CD 43	60.5[1] (4.2)[2]	53.8 (5.6)	62.6 (4.3)	41.3 (3.9)
CD 5	58.5 (3.4)	62.8 (8.2)	58.3 (3.5)	33.34 (5.1)
CD 4	28.7 (2.9)	40.38 (3.4)	48.4 (2.2)	37.8 (6.0)
CD 8	31.5 (4.2)	65.6 (5.8)	17.7 (6.9)	33.6 (7.8)
B-cell	36.2 (5.2)	36.3 (3.8)	20.5 (4.9)	31.5 (6.3)

[1] mean value of the percentage of positive cells

[2] standard error of the mean

[3] calculated as the proportion cells of that phenotype which were presumptive blasts

Figure 1 Serial sections showing lymphocyte accumulations in the lachrymal gland in an AHV-1 infected rabbit (AS 92/7). A: labelled with anti-CD43 (T-cell) B: labelled with anti-PCNA (cell multiplication)